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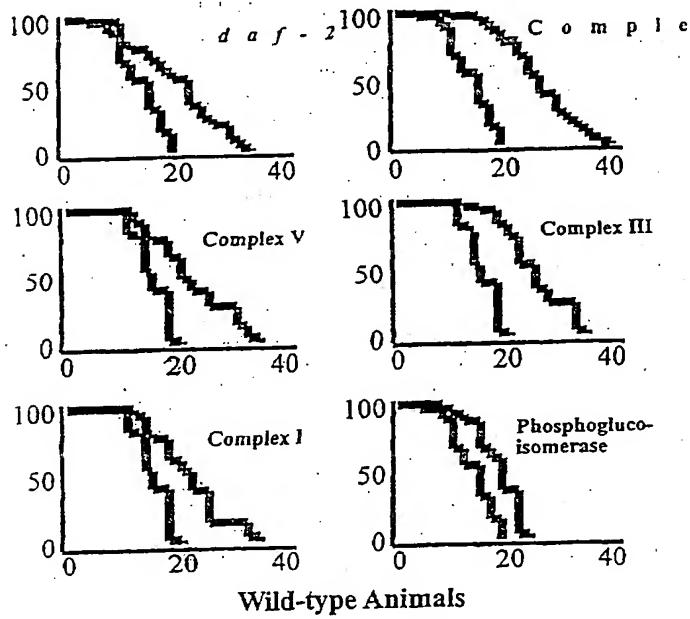
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(54) Title: EUKARYOTIC GENES INVOLVED IN ADULT LIFESPAN REGULATION

RNAi of respiratory chain and glycolysis genes increases life span

**WO 03/000861 A2**

to the use of expression profiles, markers, and compositions in diagnosis and therapy related to lifespan extension, life expectancy, and aging. The present invention also relates to gene therapy involving lifespan associated genes.

(57) Abstract: The present invention relates to regulation of adult lifespan in eukaryotes. More particularly, the present invention is directed to methods of assaying for genes, gene products, and genes in pathways controlled by such genes and gene products, using RNAi and microarray analysis, that regulate lifespan (e.g., extend or truncate adult lifespan) in eukaryotes such as invertebrates (e.g., *C. elegans*), plants, and mammals, e.g., humans. For example, the present invention is directed to genes encoding components of the mitochondrial respiratory chain and genes encoding glycolysis enzymes, which are involved in lifespan regulation, and genes and gene products in pathways controlled by such genes. Other genes and gene products identified as regulating aging and aging pathways include a gene encoding a GTPase; a transcriptional activator; novel genes: llw-1, llw-2, llw-3, and llw-4; genes encoding cytochrome P450 proteins (involved in steroid biosynthesis); a melatonin synthesis gene; genes encoding insulin and insulin-like peptides; genes encoding heat shock factors; genes encoding catalases; stress-response genes; and metabolic genes. The invention further relates to methods for identifying and using agents, including small molecule chemical compositions, antibodies, antisense nucleic acids, and ribozymes, that regulate, e.g., enhance, adult lifespan via modulation of aging associated proteins; as well as



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EUKARYOTIC GENES INVOLVED IN ADULT LIFESPAN REGULATION

CROSS-REFERENCES TO RELATED APPLICATIONS

5 The present application is related to USSN 60/300,577, filed June 22, 2001, USSN 60/301,052, filed June 25, 2001, and USSN 60/373,975, filed April 18, 2002, each herein incorporated by reference in their entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER 10 FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with government support under Grant No. NIH AG11816, awarded by the NIH. The government has certain rights in this invention.

FIELD OF THE INVENTION

15 The present invention relates to regulation of lifespan in eukaryotes. More particularly, one aspect of the present invention is directed to methods of assaying for genes, gene products, and genes in pathways controlled by such genes and gene products, using RNAi and microarray analysis, that regulate lifespan (e.g., extend or truncate adult lifespan) in eukaryotes such as invertebrates (e.g., *C. elegans*), plants, and mammals, e.g., humans.
20 For example, one aspect of the present invention is directed to genes, in particular human genes, encoding components of the mitochondrial respiratory chain and genes encoding glycolysis enzymes, which are involved in lifespan regulation, and genes and gene products in pathways controlled by such genes. Other genes and gene products identified as regulating aging and aging pathways include a gene encoding a GTPase; a transcriptional activator;
25 novel genes: *llw-1*, *llw-2*, *llw-3*, and *llw-4*; genes encoding cytochrome P450 proteins (involved in steroid biosynthesis); a melatonin synthesis gene; genes encoding insulin and insulin-like peptides; genes encoding heat shock factors; genes encoding catalases; stress-response genes; and metabolic genes. The invention further relates to methods for identifying and using agents, including small molecule chemical compositions, antibodies, antisense
30 nucleic acids, and ribozymes, that regulate, e.g., enhance, adult lifespan via modulation of aging associated proteins; as well as to the use of expression profiles, markers, and

compositions in diagnosis and therapy related to lifespan extension, life expectancy, and aging. The present invention also relates to gene therapy involving lifespan associated genes.

BACKGROUND OF THE INVENTION

Previously, classic genetic screens have been used to identify genes involved in the *C. elegans* development. In one example, inhibition of mitochondrial respiratory chain genes such as NADH ubiquinone oxidoreductase and ATP synthase in *C. elegans* larva was found to impair larval development and cause arrest in the third larval stage (see, e.g., Tsang *et al.*, *JBC* 276:33240-33246 (2001)). In other examples, classical genetic screens have been used to identify *C. elegans* genes involved in a variety of processes, including dauer formation, and embryonic development. Some of these genes, for example the *daf-2* and *daf-16* genes, have been implicated in the regulation of lifespan see, e.g., Kenyon *et al.*, *Nature* 366:461-464 (1993); Morris *et al.*, *Nature* 382:536-539 (1996); Kimura *et al.*, *Science* 277:942-946 (1997); Paradis *et al.*, *Genes Dev.* 12:2488-2498 (1998); Paradis *et al.*, *Genes Dev.* 13:1438-1452 (1999); Off & Ruvkun, *Mol. Cell* 2:886-893 (1998); Guarante & Kenyon, *Nature* 408:255-262 (2000); Ogg *et al.*, *Nature* 389:994-999 (1997); and Lin *et al.*, *Science* 278:1319-1322 (1997).

Classical genetic screens are frequently time consuming, both in identification of interesting mutants and in cloning a gene associated with a mutation. Classical genetic screens can include labor intensive backcrosses to eliminate mutations unlinked to the phenotype of interest. Classical genetic screens also may require the extra step of cloning the gene of interest, by complementation of the mutation.

Many different genes likely regulate the process of aging in eukaryotes and their identification will aid in understanding the process. Regulation of biological processes is frequently conserved between divergent organisms. For example, cell cycle proteins and their mechanism of action are conserved between organisms as divergent as yeast and humans. Thus, regulatory mechanisms identified in a genetically tractable organism can be used to predict and identify homologous genes and gene products that regulate similar biological processes in higher eukaryotes. However, even in genetically tractable organisms, such as *C. elegans*, classical genetic methods are frequently labor intensive and cumbersome for identification of interesting mutants and for isolation of a gene of interest. The present invention solves these and other problems.

SUMMARY OF THE INVENTION

The present invention provides, for the first time, methods of assaying for aging associated genes and proteins using RNAi, as well as aging associated genes and proteins so identified. Double-stranded RNA libraries are administered to a test organism or 5 cell, e.g., *C. elegans*. For administration to *C. elegans*, the RNAi library can be expressed in a bacterial cell and fed to the *C. elegans*. Age-associated mutants (those that live longer or age faster) are identified by measuring lifespan increase or decrease, or an age-associated parameter such as stress resistance or a Nomarski analysis indicia, e.g., yolk accumulation, loss of ability to chew and expel (distended oral and anal cavities), necrotic cavities in tissue, 10 curdled appearing tissue, and germ cell appearance (graininess, large, well separated nuclei, fewer nuclei, and cavities).

Other methods can be used to identify age-associated genes and gene products, including microarray analysis, database profiling with known age-associated genes, mammalian complementation assays, yeast two hybrid assays, immunoprecipitation, etc.

15 The use of dsRNA has identified a number of genes and gene products which, when inhibited by dsRNA, results in longer lifespans, e.g., phosphoglucose isomerase, cytochrome C1, NADH oxidoreductase, ATP synthase, cytochrome C oxidase, a GTPase, and four novel genes, *llw1-4* (see also Tables 5 and 6). Thus, compounds that inhibit such genes and gene products, as well as genes in a pathway controlled by such genes and gene 20 products, would be useful for increasing lifespan and modulating the aging process in eukaryotes, e.g., plants, mammals, humans. The genes identified herein include any mammalian or human homologs thereof.

In addition, the use of dsRNA has identified genes and gene products which, 25 when inhibited by dsRNA, results in a shorter lifespan, e.g., heat shock protein or heat shock factor (HSF) (see also Table 5 and Table 6). Overexpression of HSF has also been shown to increase lifespan in *C. elegans*. Thus, compounds that activate heat shock proteins or genes such as HSF-1 or HSF targets, or genes or gene products controlled by HSF, or genes that respond to stress and activate HSF would be useful for increasing lifespan and modulating the aging process in eukaryotes, e.g., plants, mammals, humans. In one example, compounds 30 such as zinc finger proteins (naturally occurring or recombinant) can be used to bind to HSF binding regions on genes and alter expression of HSF-controlled genes (see, e.g., U.S. 5,789,538 and 6,242,568).

Microarrays were used to analyze gene expression profiles in *daf-2* and *daf-16* mutants and identify genes and gene products involved in lifespan regulation. The expression

of a number of genes varied (by over- or under-expression) in long-lived (*daf-2*) or short-lived (*daf-16* and *daf-16; daf-2*) *C. elegans* mutants (see Table 5). The activity of genes identified using microarray analysis was then modulated using RNAi (Table 6). The genes thus identified include hormones that activate the *daf-2* pathway, several encoding 5 cytochrome P450 proteins (involved in steroid biosynthesis), the melatonin synthesis gene, insulin and insulin-like peptides, heat shock factors, catalases, stress-response genes, and metabolic genes. These genes and gene products, and genes and gene products controlled by these genes (e.g., steroid hormones, melatonin) are therefore useful for developing drugs to regulate aging in eukaryotes, e.g., plants, mammals, humans. In addition, these genes can be 10 used as markers for the insulin/IGF system activity, as markers for the aging process, and as markers that indicate the likely longevity of an individual.

In one aspect of the invention, nucleic acids from *C. elegans* and corresponding mammalian genes (e.g., human) encoding glycolysis proteins, e.g., phosphoglucose isomerase, and mitochondrial respiratory chain proteins, e.g., cytochrome C1 component of complex III (CYC1), NADH oxidoreductase (NUO2), ATP synthase (ATP3, a member of the ATP synthase delta family), and cytochrome C oxidase (CCO1), are provided, as are nucleic acids from *C. elegans* and corresponding mammalian genes encoding a GTPase associated with aging; a transcriptional activator, heat shock factors, e.g., HSF-1; novel proteins LLW-1, LLW-2, LLW-3, and LLW-4; cytochrome P450 proteins, a melatonin synthesis gene, insulin and insulin-like peptides, heat shock factors, catalases, stress-response genes, and other genes listed in Tables 5 and 6. In another aspect, the present invention provides nucleic acids, such as probes, antisense oligonucleotides, and ribozymes, that hybridize to glycolysis genes, e.g., phosphoglucose isomerase (GPI-1); mitochondrial respiratory chain genes, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and 20 cytochrome C oxidase; a GTPase associated with aging; a transcriptional activator, heat shock factors, e.g., HSF-1; novel genes *llw-1*, *llw-2*, *llw-3*, and *llw-4*; cytochrome P450 proteins, a melatonin synthesis gene, insulin and insulin-like peptides, heat shock factors, catalases, stress-response genes, and other genes listed in Tables 5 and 6.

In another aspect, the invention provides expression vectors and host cells comprising nucleic acids encoding glycolysis proteins, e.g., phosphoglucose isomerase; mitochondrial respiratory chain proteins, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and cytochrome C oxidase; a GTPase associated with aging; a transcriptional activator, heat shock factors, e.g., HSF-1; novel genes *llw-1*, *llw-2*, *llw-3*, and *llw-4*; cytochrome P450 proteins, a melatonin synthesis gene, insulin and insulin-like peptides, heat

shock factors, catalases, stress-response genes, and other genes listed in Tables 5 and 6. In another aspect, the present invention provides glycolysis proteins, e.g., phosphoglucose isomerase; mitochondrial respiratory chain proteins, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and cytochrome C oxidase; a GTPase associated with aging; a transcriptional activator, the heat shock factor; novel proteins LLW-1, LLW-2, , LLW-3, 5 LLW-4; cytochrome P450 proteins, a melatonin synthesis protein, insulin and insulin-like peptides, heat shock factors, catalases, stress-response proteins, and other gene products listed in Tables 5 and 6 and antibodies thereto.

In another embodiment, the invention provides heterologous constructs 10 comprising an age-associated gene as described herein, and a heterologous sequence such as a regulatory region, a reporter gene, a purification tag, e.g., for production of a fusion protein, for purification of a gene product, for more efficient expression of the gene or gene product, or for regulated expression of the gene.

In one embodiment, methods known to those of skill in the art such as RT- 15 PCR, northern, Southern analysis, cDNA and genomic library cloning, etc. can be used to identify eukaryotic orthologs, e.g., invertebrate, vertebrate, plant, mammalian, and human orthologs, of the age-associated proteins provided herein. In another embodiment, computer sequence analysis can be used to identify orthologs. Such methods optionally include the step of assessing an age associated parameter in a cell in which the suspected ortholog is 20 perturbed.

In one embodiment, endogenous or recombinant gene products of the age 25 associated genes described herein are purified using the methods described herein, to at least about 50% purity, preferably 60%, 70%, 80%, 90% or higher purity. In another embodiment, the present invention provides a reaction mixture comprising an age-associated protein and another component such as a test compound, an antibody, a peptide, etc.

In another aspect, the present invention provides a method for identifying a compound that modulates adult aging, the method comprising the steps of: (i) contacting the compound with a glycolysis protein, e.g., phosphoglucose isomerase, or a mitochondrial respiratory chain protein, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and 30 cytochrome C oxidase or a GTPase associated with aging; a novel proteins LLW-1, LLW-2, LLW-3, LLW-4; and (ii) determining the functional effect, e.g., lifespan effect or another age-associated parameter of the compound upon the polypeptide.

In another aspect, the present invention provides a method for identifying a compound that modulates adult aging, the method comprising the steps of: (i) contacting the

compound with protein encoded by a gene listed in Table 5 or 6; and (ii) determining the functional effect, e.g., lifespan effect or another age-associated parameter of the compound upon the polypeptide.

In another aspect, the present invention provides a method for identifying a compound that modulates adult aging, the method comprising the steps of: (i) contacting the compound with a heat shock factor and (ii) determining the functional effect, e.g., lifespan effect or another age-associated parameter, of the compound upon the polypeptide.

In one embodiment, the functional effect is a physical effect or a chemical effect. In another embodiment, the functional effect is a phenotypic effect. In one embodiment, the polypeptide is expressed in a eukaryotic host or host cell, e.g., *C. elegans*. In another embodiment, the functional effect is determined by measuring longevity, average lifespan, or mean lifespan of an organism contacted with a compound. In one embodiment, the functional effect is determined by measuring enzymatic activity. In one embodiment, the functional effect is determined by measuring transcriptional activation. In one embodiment, the organism is *C. elegans*. In another embodiment, the organism is mammalian host or cell, e.g., a mouse, a rat, a guinea pig, a monkey, or a human.

In another embodiment, compounds that modulate aging are identified using computer programs that model age-associated protein structure and determining compounds that bind or interact with the modeled protein. Optionally, the effect of the compound can be validated by examining its effect on a cell or organism expressing the modeled age-associated protein.

In another embodiment, the method comprises providing a sequence comprising an age-associated protein, altering the sequence, e.g., by mutagenesis, and assaying the protein encoded by the altered sequence.

In another aspect, the present invention provides a method of modulating lifespan in a subject, the method comprising the step of contacting the subject with an therapeutically effective amount of a compound identified using the methods described herein, e.g., a compound such as antimycin. In one embodiment, the subject is *C. elegans*. In another embodiment, the subject is a mammalian subject, e.g., a mouse, a rat, a guinea pig, a monkey, or a human. In one embodiment, the subject is a plant. In one embodiment, the compound is antimycin or an analog thereof.

In another aspect, the present invention provides a method of detecting the presence of a lifespan associated protein described herein, and the genes encoding such proteins in eukaryotic tissue, the method comprising the steps of: (i) isolating a biological

sample; (ii) contacting the biological sample with a specific reagent that selectively associates with the protein of choice; and, (iii) detecting the level of specific reagent that selectively associates with the sample.

In one embodiment, the specific reagent is selected from the group consisting
5 of: antibodies, oligonucleotide primers, and nucleic acid probes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows RNAi of respiratory chain and glycolysis genes increasing lifespan.

10 Figure 2 shows *daf-2(e1370)* worms treated with dsRNAs of respiratory and glycolytic genes.

Figure 3 shows antimycin increases lifespan.

Figure 4 shows *daf-16(mu86)* mutant worms treated with dsRNAs of respiratory and glycolytic genes.

15 Figure 5 shows certain genes identified using microarray analysis that are upregulated in *daf-2(-)* worms.

Figure 6 shows that hsp-12.6 RNAi reduces *daf-2* longevity.

Figure 7 shows that mtl-1 RNAi reduces *daf-2* longevity.

Figure 8 shows that cytochrome P450 shortens *daf-2* lifespan.

20 Figure 9 shows certain genes identified using microarray analysis that are downregulated in *daf-2* mutants.

Figure 10 shows that RNAi of vitellogenins increases lifespan.

Figure 11 shows that RNAi of ASMLT (melatonin synthesis gene) prolongs lifespan.

25 Figure 12 shows certain genes that modulate lifespan.

DETAILED DESCRIPTION OF THE INVENTION

INTRODUCTION

Double-stranded RNA-mediated interference (RNAi) provides a sequence specific mechanism for inhibiting gene expression in *C. elegans* (see, e.g., Fire *et al.*, *Nature* 391:806-811 (1998) and WO 99/32619)). This technique is also useful for functional genomics analysis of *C. elegans* genes (see, e.g., Fraser *et al.*, *Nature* 408:325-330 (2000); Kamath *et al.*, *Genome Biol.* 2:RESEARCH0002 (2000)).

Surprisingly, RNAi can be used to identify genes and gene products involved in lifespan and aging mechanisms in adults, by feeding bacteria expressing a dsRNA library over long-term time periods to *C. elegans* and measuring, e.g., mean and median lifespan increase or decrease in adults. Such assays can be used, in addition, to determine if two or 5 more genes function together in a similar aging pathway, to determine if gene function is cell autonomous, and to determine if drug compounds known to alter the aging process function in the same or different pathways. Microarray assays can also be used to identify genes and gene products involved in lifespan and aging (see, e.g., Table 5 and Table 6).

Microarray and RNAi assays are also useful in combination, e.g., by 10 identifying a gene or gene product involved in lifespan using RNAi and then examining its expression pattern in old and young adults using microarray analysis, or by identifying a differentially expressed gene or gene product involved in lifespan using microarray analysis and then examining the effects of gene or gene product inhibition using RNAi. Microarray analysis can also be performed on animals treated with RNAi of genes identified using 15 microarray analysis.

Using the technique of double-stranded RNA inhibition (RNAi), two types of metabolic genes and gene products have been found to regulate the adult lifespan of a model eukaryote, *C. elegans*. Inhibition of genes and gene products that function in the mitochondrial respiratory chain, including genes encoding cytochrome C1, cytochrome C oxidase and ATP synthetase, lengthen lifespan, as does inhibition of a gene that functions in glycolysis, glucose phosphate isomerase. However, these perturbations produce different phenotypes. Inhibition of respiratory chain components cause a Clk (clock) phenotype, including slow growth to adulthood, and, in adults, slow movement, and decreased pumping and defecation rates. In contrast, inhibition of glucose phosphate isomerase extends adult 20 lifespan without producing a Clk phenotype. Together these findings indicate that at least two distinct pathways can regulate lifespan in response to changes in metabolic gene activities in eukaryotes. These RNAi-mediated phenotypes are examples of "reverse phenotypes," i.e., suppression or inhibition of the gene lengthens lifespan. For RNAi interventions that decrease lifespan, restoring gene expression should restore a normal 25 lifespan. Therefore, under- and over-expression of lifespan genes, gene products and pathways, as well as activation or inhibition of lifespan gene, gene products, and pathways, 30 can be used to modulate aging in a subject.

The present invention therefore provides nucleic acids from *C. elegans* encoding cytochrome C1 component of complex III, gene C54G4.8 (CYC1, SEQ ID NO:1,

Accession No. CAA99820.1, which is 50% identical to human ortholog CYC1); NADH oxidoreductase, gene T10E9.7 (NUO2, SEQ ID NO:2, Accession No. AAB522474.1, which is 56% identical to human ortholog NDUFS3, Accession No. NM_004551); ATP synthase (delta family), gene F27C1.7 (ATP3, SEQ ID NO:3, Accession No. AAB37654.1, 43% identical to human ortholog ATP50); and cytochrome C oxidase, gene F26E4.9 (CCO1, SEQ ID NO:4, Accession No. CAB03002.1, 35% identical to human ortholog COX5B, Accession No. NM_001862), which are protein components of the mitochondrial respiratory chain. The present invention also provides nucleic acids from *C. elegans* encoding glucose phosphate isomerase, gene Y87G2A.8 (GPI-1, SEQ ID NO:5, Accession No. CAB60430.1, 68% identical to human ortholog GPI, Accession No. NM_000175). The present invention also provides gene and gene products shown in Tables 5 and 6, and human homologs thereof. The present invention also provides homologs of these conserved proteins that are found in mammals, such as humans, and which are known to those of skill in the art. The invention therefore provides methods of screening for compounds, e.g., small molecules, antibodies, antisense molecules, and ribozyme, that are capable of modulating lifespan in adult eukaryotes, in particular, in mammals, e.g., for lifespan enhancement and treatment of premature aging.

Furthermore, it was discovered that administration of antimycin, a mitochondrial respiration inhibitor, substantially increased lifespan in the model *C. elegans*.

The present invention includes other genes and gene products that regulate the aging process in *C. elegans*. One such gene, encodes a GTPase, gene T23H2.5; (SEQ ID NO:6, Accession No. AAC48200.1). Inhibition of GTPase results in a longer lifespan.

Four other genes and gene products that regulate aging in *C. elegans* are also provided: *llw-1*, gene Y54G11A.8 (SEQ ID NO:7, Accession No. CAA22452); *llw-2*, gene F59E12.10 (SEQ ID NO:8, Accession No. AAB54251); and *llw-3*, gene Y48E1B.1 (SEQ ID NO:9, Accession No. CAB07688). Also included is *llw4*, gene F45H10.4 (SEQ ID NO:10, Accession No. CAB04386). Inhibition of these genes results in a Long-Lived Worm (*llw*) phenotype.

Also using the technique of double stranded RNA inhibition, a gene encoding a heat shock factor (HSF) or heat shock protein (HSP) was found to regulate aging in *C. elegans*. Inhibition of the HSF gene and gene product resulted in worms that display the objective characteristic of aging prematurely (described below) and have short lifespans compared to control worms. HSF is a transcriptional activator of stress related genes. The present invention provide nucleic acids from *C. elegans* encoding HSF-1, gene Y53C10A.12

(SEQ ID NO:11, Accession No. CAA22146). As described above, for RNAi treatments that decrease lifespan, restoring gene expression should restore a normal lifespan. Furthermore, overexpression of this gene in *C. elegans* extends lifespan.

It has been discovered that old worms have a number of characteristic features
5 that are highly recognizable and diagnostic when the animals are viewed with Nomarski optics. These characteristics allow for a determination of whether or not a worm is aging prematurely, aging normally, aging slowly, or not aging at all, e.g., has a mutation that causes a sick rather than an aging phenotype. Thus, these objective characteristics can be used as an assay, in combination with RNAi, for genes, gene products, and drugs involved in lifespan regulation.
10 The present invention therefore also provides genes, identified using RNAi, that regulate the aging process in adult eukaryotes.

As described above, the present invention provides a method of using RNAi to identify genes and gene products involved in lifespan regulation, by screening, e.g., for increased or decreased mean and median lifespan. The present invention also demonstrates
15 that Nomarski microscopy provides a powerful way to monitor the aging process in cells and tissues of *C. elegans*. This technique permits an objective of the quality of cells and tissues that make up most of the body mass. It was found that the tissues of aging animals have a very characteristic appearance, using a number of objective criteria, e.g., yolk accumulation, loss of ability to chew and expel (distended oral and anal cavities), bacterial packing in the
20 intestine (constipation), necrotic cavities in tissue, curdled appearing tissue, and germ cell appearance (graininess, large, well separated nuclei, fewer nuclei, and cavities). These characteristics allow for a determination of whether or not a worm is aging prematurely (progeria), aging normally, aging slowly, or not aging at all, e.g., has a mutation that causes a sick rather than an aging phenotype. Thus, these objective characteristics can be used, as an
25 assay for genes and drugs involved in lifespan regulation. In one embodiment, the characteristics are used to evaluate an organism or cell that is treated with RNAi. In another embodiment, the characteristics are used to evaluate an organism or cell that is genetically mutated. Using the technique of double stranded RNA inhibition, a gene encoding Heat Shock Factor (HSF) was found to prevent premature aging in *C. elegans*.

30 In one aspect, the invention features a method that includes: (1) providing a library of nucleic acids, each nucleic acid of the library comprising a segment of a gene of an organism; and (2) for each member of the library, (a) generating double-stranded RNA (dsRNA) from the respective member of the library, (b) providing the dsRNA to a cell or one or more cells of an organism to provide a dsRNA treated cell or a dsRNA treated organism,

and (b) monitoring an age-associated parameter of the dsRNA treated cell or the dsRNA treated organism. Exemplary dsRNA-treated organisms include a nematode, *C. elegans*, an organism other than a nematode, *Drosophila*, zebrafish, and a mammal, e.g., a mouse. The providing of dsRNA can include feeding the organism the dsRNA or a bacterium that expresses the dsRNA. Exemplary dsRNA-treated cells include a *Drosophila* cell or a mammalian cell, e.g., a murine, canine, bovine, primate, or human cell. The cell can be culture *in vitro*. The age-associated parameter can be any age-associated parameter, e.g., a parameter described herein such as lifespan.

The method can be repeated for a cohort of dsRNA treated organisms and the age-associated parameter can include a survival curve for the cohort. The method can further include, for each member of the library indicated as affecting lifespan of the organism, evaluating abundance of an RNA or a protein corresponding to indicated library member or an RNA or protein corresponding to a gene listed in Tables 5 and 6. The abundance can be evaluated, for example, in a defined set of cells or in organisms of defined age. The abundance can be evaluated using an array, e.g., a nucleic acid microarray. The method can include other features described herein.

In another aspect, the invention features a method that includes: evaluating a relationship between presence or abundance for each species of a plurality of RNA or protein species with respect to age of a cell or organism; producing or delivering a double-stranded RNA to a cell or one or more cells of an organism, the RNA corresponding to a species whose presence or abundance is correlated with age; and monitoring an age-associated parameter of the cell or the one or more cells or the organism. Evaluating the relationship can include transcriptional profiling using a nucleic acid array. The plurality of RNA or protein species can include one or more species corresponding to a gene listed in Tables 5 and 6. The plurality can include at least 10, 20, 40, 50, 60, 70% or 100% of the genes listed in Tables 5 and 6. In one embodiment, the organism is an invertebrate. In another embodiment, the organism is a vertebrate, e.g., a mammal. Similarly, a cell can be a cell of a vertebrate (e.g., a mammal, e.g., a human) or an invertebrate, e.g., a nematode.

In another aspect, the invention features a method that includes: contacting a test compound to a living or biochemical system that includes a target protein selected from the group consisting of: a respiratory chain component, a heat shock promoter activator (e.g., an HSF protein or is an artificial chimeric transcription factor), LLW-1, LLW-2, LLW-3, LLW-4, a small GTPase, a protein encoded by a gene listed in Table 5 and 6, and mammalian (e.g., human) homologues thereof; and evaluating a property associated with the target

protein. The method can be used, e.g., to evaluating a test compound, e.g., for an effect on lifespan or a lifespan-related process. In one embodiment, the target protein is a *C. elegans* protein, e.g., a protein that includes SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11. In another embodiment, the target protein is a mammalian protein.

5 The living or biochemical system can be a mammalian or non-mammalian system. In one embodiment, the system includes a cell extract, e.g., a lysate or fraction of a cell, e.g., a membrane preparation, a cytoplasmic preparation, or a partially or completely purified preparation. In another embodiment, the system includes isolated mitochondria, e.g., a cell extract or fraction enriched in mitochondria. In still another embodiment, the system
10 includes a living cell, e.g., cultured cells, e.g., primary cell or transformed cells. In yet another embodiment, the system includes a living organism. The organism includes a cell that can express the target protein.

15 The method can further include contacting the test compound to an organism (e.g., *C. elegans*, *Drosophila*, or a mammal, e.g., a mouse) and evaluating an age-associated parameter of the organism.

20 Exemplary properties include a catalytic parameter; structural conformation; post-translational modification; redox state; physical interaction of the target protein with another protein; metabolite formation or consumption; subcellular localization of the target protein; in vivo half-life of the target protein or target protein activity; transcription of a gene encoding the target protein or translation of the target protein.

25 In one embodiment, the catalytic parameter describes the catalytic properties of an enzyme, other than the target protein. For example, the target protein is a substrate of the enzyme. In one embodiment, the post-translational modification is a modification of the target protein. In another embodiment, the post-translational modification is catalyzed by the target protein. Exemplary post-translational modifications include phosphorylation, ubiquitination, methylation, acetylation/deacetylation, geranylgeranylation, farnesylation, or proteolytic modification. In one embodiment, where the property relates to metabolite production or consumption, the metabolite can be a direct substrate or direct product of a reaction catalyzed or effected by the target protein. In another embodiment, where the
30 property relates to metabolite production or consumption, the metabolite can be an indirect substrate or indirect product of a reaction catalyzed or effected by the target protein.

 A culture cell used in the method can include a heterologous nucleic acid that encodes and expresses the target protein. The method can further include assessing whether the test compound directly interacts with the target protein.

In one embodiment, the target protein is operably linked to a reporter protein and the evaluating comprises evaluating the reporter protein.

In one embodiment, the target protein is a cytochrome, and the property is redox state of the cytochrome.

5 In another aspect, the invention features a method that includes providing a nematode in which activity of a target protein is reduced in the organism by RNA interference; expressing a gene encoding a protein (e.g., candidate protein) that is heterologous to the organism; and evaluating an age associated parameter of the organism. The method can further include, prior to the evaluating, contacting the organism with a test 10 compound. The candidate protein can be, for example, a mammalian protein. The candidate protein can be, for example, a respiratory chain component. The method can include other features described herein.

15 In another aspect, the invention features a method that includes: providing a cell in which activity of a target protein is reduced in the organism by RNA interference; expressing a gene encoding a protein (e.g., candidate protein) that is heterologous to the organism; and evaluating an age associated parameter of the organism. The method can include other features described herein.

20 In still another aspect, the invention features a method that includes providing a cell or an organism (e.g., a nematode) in which activity of a target protein is reduced in the cell or one or more cells of the organism by RNA interference; contacting the organism or the cell with a test compound; and evaluating an age associated parameter of the organism.

25 In another aspect, the invention features a method that includes assessing an age-related parameter of a nematode that (1) expresses a heterologous gene in at least some cells; and (2) is deficient in at least some cells for an endogenous activity provided by a respiratory chain component. In one embodiment, the heterologous gene is from a non-nematode species, e.g., a mammalian species. In a related embodiment, the heterologous gene encodes a variant of a mammalian protein, the variant having between one and ten substitutions, insertions, or deletions. The heterologous gene can encode a domain of at least 30 amino acids from a mammalian protein or a variant thereof, having between one and six 30 substitutions, insertions, or deletions.

In one embodiment, the endogenous activity is provided by a respiratory chain component (e.g., cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5)), a GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2

(SEQ ID NO:8), LLW-3 (SEQ ID NO:9); LLW-4 (SEQ ID NO:10); HSF-1 (SEQ ID NO:11), or a protein listed in Table 5 or 6.

The method can include contacting the organism with a test compound, e.g., prior to the assessing. The method can include other features described herein.

5 In another aspect, the invention features a method of characterizing a protein, the method includes: providing a nucleic acid that encodes a protein having a subject amino acid sequence that contains at least one substitution, insertion, or deletion relative to a reference amino acid sequence, wherein the subject amino acid sequence and the reference amino acid sequence are at least 70% identical; expressing the nucleic acid in a culture cell or
10 in an invertebrate cell; and evaluating an age-associated parameter of the cell, or an organism that includes the cell. The reference amino acid sequence can be selected from a sequence described herein, e.g., one of the following sequences cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1
15 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), LLW-4 (SEQ ID NO:10); HSF-1 (SEQ ID NO:11), the amino acid sequences of proteins listed in Table 5 and 6, and human homologues.

In another aspect, the invention features a *C. elegans* nematode that (1) expresses a heterologous gene in at least some cells, the heterologous gene encoding a heterologous protein (e.g., a protein described herein (e.g., a mammalian gene described herein) that is non-identical to a corresponding endogenous protein, or a functional domain thereof; and (2) is deficient in at least some cells for an endogenous activity provided by the corresponding endogenous protein. For example, the nematode can (1) express a heterologous gene in at least some cells, the heterologous gene encoding a respiratory chain component that is non-identical to the corresponding endogenous respiratory chain component, or a functional domain thereof; and (2) be deficient in at least some cells for an endogenous activity provided by the corresponding endogenous respiratory chain component.
25

In one embodiment, the heterologous gene encodes a mammalian protein. In another embodiment, the heterologous gene encodes a variant of a mammalian protein, the variant having between one and ten substitutions, insertions, or deletions. For example, the heterologous gene encodes at least a domain of at least 30 amino acids from a mammalian protein or a variant thereof, having between one and six substitutions, insertions, or deletions. The deficiency can be mediated by dsRNA, e.g., by RNA interference.
30

The invention also features a method that includes assessing an age-associated parameter of a nematode described herein, e.g., a nematode described above. In one embodiment, the method further includes, prior to the assessing, contacting the nematode to a test compound.

5 In another aspect, the invention features a *C. elegans* nematode that (1) is deficient in at least some cells for an endogenous activity, the deficiency generated by dsRNA in the cells, and (2) has an average lifespan of at least 24, 26, or 28 days (e.g., in the N2 background) or an average lifespan of at least 25% greater than the average lifespan of an otherwise identical nematode not contacted with the dsRNA. The nematode is such that, 10 absent the deficiency, the nematode has an average lifespan of less than 22, 20, 18, or 16 days. In one embodiment, the nematode has functional genes for dauer pathway and/or functional genes for *clk-1*, *gro-1*, and/or another gene described herein. For example, the nematode can be wild-type with respect to a laboratory standard, e.g., the N2 background or another background. The dsRNA may include a strand that is complementary to a nucleic acid encoding a protein described herein, e.g., comprising an amino acid sequence selected from the group consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9); LLW-4 (SEQ ID NO:10); and 15 HSF-1 (SEQ ID NO:11), proteins listed in Table 5 and 6, and human homologues corresponding to all of the above.

20

In another aspect, the invention features a method that includes: providing a nematode described herein, e.g., a nematode described above; introducing a heterologous gene that encodes a polypeptide into the nematode; expressing the heterologous gene in the 25 nematode or a progeny of the nematode under conditions wherein the polypeptide is produced; and monitoring an age-associated parameter of the nematode or the progeny of the nematode. The heterologous gene can be, e.g., a nematode gene or a mammalian gene.

In another aspect, the invention features a method includes: providing a library 30 of a test agents; contacting each test agent of the library to cells; evaluating expression of a HSF-regulated gene in the contacted cells; for each test agent that alters the expression of a HSF-regulated gene, contacting a test organism with the test compound; and evaluating an age-associated parameter of the test organism. Many genes regulated by HSF are known or can be readily identified. See, e.g., GuhaThakurta *et al.* *Genome Res* 12(5):701-12 (2002) and Christians *et al.* *Crit Care Med* 2002 Jan;30(1 Suppl):S43-50 (2002).

In another aspect, the invention features a nucleic acid vector that includes (1) a coding sequence encoding an amino acid sequence comprising SEQ ID NO:7, 8, 9, or 10 or a fragment thereof; and (2) one or more of the following: (a) a promoter that is operably linked and heterologous to the coding sequence, and (b) a second coding sequence encoding a reporter protein or protein tag, the second coding sequence being operably linked and heterologous to the coding sequence. The invention also features a dsRNA (e.g., an isolated dsRNA) that comprises a nucleic acid segment that is complementary to a *C. elegans* gene that encodes SEQ ID NO:7, 8, 9, or 10, wherein the dsRNA, when administered to a *C. elegans* or cell thereof, causes an extension of lifespan of at least 20%. In one embodiment, 5 the dsRNA is not complementary to a homologue (e.g., a homologue described herein) of the *C. elegans* gene that encodes SEQ ID NO:7, 8, 9, or 10. The invention also provides a method that includes providing (e.g., feeding) an adult nematode a dsRNA described herein. 10 The invention also features an antibody that binds to SEQ ID NO:7, 8, 9, or 10. In a related aspect, the invention features a nucleic acid vector that includes (1) a coding sequence 15 encoding an amino acid sequence comprising to a gene listed in Table 5 or 6; and (2) one or more of the following: (a) a promoter that is operably linked and heterologous to the coding sequence, and (b) a second coding sequence encoding a reporter protein or protein tag, the second coding sequence being operably linked and heterologous to the coding sequence.

The invention also features a method that includes: providing a cell, organism, 20 or biochemical system that includes a subject protein described herein (e.g., a protein comprising an amino acid sequence selected from the group consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9); 25 LLW-4 (SEQ ID NO:10); and HSF-1 (SEQ ID NO:11), and proteins described in Tables 5 and 6, or human homologues thereof); contacting an antibody that binds to the subject protein or antigen-binding fragment thereof to the cell or the organism; and evaluating an age-associated parameter of the cell, organism, or biochemical system.

30 DEFINITIONS

As is known to those of skill in the art, "oxidative phosphorylation" or "mitochondrial respiratory chain protein" and "glycolysis" proteins are involved in metabolic energy pathways that are universal in eukaryotic biological systems. The terms "oxidative phosphorylation or mitochondrial respiratory chain protein," e.g., cytochrome C1 (SEQ ID

NO:1 Accession No. CAA99820.1); NADH oxidoreductase (SEQ ID NO:2 Accession No. AAB522474.1); ATP synthase (SEQ ID NO:3 Accession No. AAB37654.1); and cytochrome C oxidase (SEQ ID NO:4 Accession No. CAB03002.1); or a "glycolysis protein," e.g., phosphoglucose isomerase, hexose isomerase, or glucose phosphate isomerase (SEQ ID NO:5 Accession No. CAB60430.1); or "GTPase" e.g., (SEQ ID NO:6 , Accession No. AAC48200.1); or "heat shock factor," e.g. HSF-1 (SEQ ID NO:11, Accession No. CAA22146); or "Long-lived worm protein," e.g. LLW-1 (SEQ ID NO:7, Accession No. CAA22452), LLW-2 (SEQ ID NO:8, Accession No. AAB54251), LLW-3 (SEQ ID NO:9, Accession No. CAB07688), LLW-4 (SEQ ID NO:10, Accession No. CAB04386), or a nucleic acid encoding a "mitochondrial respiratory chain protein, e.g., cytochrome C1, cytochrome C oxidase, NADH oxidoreductase, and ATP synthase" or a "glycolysis protein, e.g., phosphoglucose isomerase," or a "GTPase" or a "heat shock factor" or a "long-lived worm protein, e.g., llw-1, llw-2, llw-3, llw-4" as well as the genes and gene products listed in Table 5 and Table 6 refer to eukaryotic and mammalian nucleic acids and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs that: (1) have an amino acid sequence that has greater than about 50% amino acid sequence identity, preferably 60%, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to an amino acid sequence encoded by the *C. elegans* genes provided herein; (2) bind to antibodies, e.g., polyclonal antibodies, raised against an immunogen comprising an amino acid sequence of a *C. elegans* mitochondrial respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein, or gene product listed in Table 5 or 6 provided herein, and conservatively modified variants thereof; (3) specifically hybridize under moderately stringent hybridization conditions to an anti-sense strand corresponding to a nucleic acid sequence encoding an *C. elegans* mitochondrial respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein or gene listed in Table 5 or Table 6 as provided herein, and conservatively modified variants thereof; (4) have a nucleic acid sequence that has greater than about 50%, preferably greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or higher nucleotide sequence identity, preferably over a region of at least about 25, 50, 100, 200, 500, 1000, or more nucleotides, to a *C. elegans* mitochondrial respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein as provided herein; and/or (5) sequences that genetically complement the *C. elegans* loss of function of a mitochondrial

respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein or gene listed in Table 5 or Table 6, as provided herein. A polynucleotide or polypeptide sequence is typically from a eukaryote, e.g., an invertebrate, vertebrate, or plant, preferably a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster, 5 cow, pig, horse, sheep, or any mammal. The nucleic acids and proteins of the invention include both naturally occurring or recombinant molecules. The sequence of the *C. elegans* genome can be found in *Science* 282:2012-2018 (1998).

The definition explicitly includes the human or mammalian homologues or counterparts of each *C. elegans* aging associated gene or protein described herein, e.g., 10 cytochrome C1, gene C54G4.8 (SEQ ID NO:1, Accession No. CAA99820.1), NADH oxidoreductase, gene T10E9.7 (SEQ ID NO:2, Accession No. AAB522474.1), ATP synthase, gene F27C1.7 (SEQ ID NO:3, Accession No. AAB37654.1), cytochrome C oxidase, gene F26E4.9 (SEQ ID NO:4, Accession No. CAB03002.1), GTPase, gene T23H2.5; (SEQ ID NO:6 , Accession No. AAC48200.1) *llw-1*, gene Y54G11A.8 (SEQ ID NO:7, Accession No. 15 CAA22452); *llw-2*, gene F59E12.10 (SEQ ID NO:8, Accession No. AAB54251); *llw-3*, gene Y48E1B.1 (SEQ ID NO:9, Accession No. CAB07688); *llw-4*, gene F45H10.4 (SEQ ID NO:10, Accession No. CAB04386); HSF-1, gene Y53C10A.12 (SEQ ID NO:11, Accession No. CAA22146), and the genes and gene products listed in Tables 5 and 6.

The phrase "functional effects" in the context of assays for testing compounds 20 that modulate activity of aging associated genes and proteins includes the determination of a parameter that is indirectly (e.g., upstream or downstream biochemical or genetic effects) or directly under the influence of aging associated proteins, e.g., a chemical or phenotypic effect, such as the ability to increase or decrease lifespan (see, e.g., Kenyon *et al.*, *Nature* 366:461-464 (1993); Hsin & Kenyon, *Nature* 399:362-366 (1999); Apfeld & Kenyon, *Cell* 25 95:199-210 (1998); and Lin *et al.*, *Nature Genet.* 28:139-145 (2001)) or, e.g., a physical effect such as ligand, cofactor or substrate binding or inhibition of ligand, cofactor or substrate binding. A functional effect therefore includes ligand, cofactor and substrate binding activity; changes in gene expression and gene expression levels in cells; changes in post transcriptional modification of a protein, e.g., phosphorylation or glycosylation; reporter 30 gene or marker expression; changes in abundance and cellular localization; enzymatic activity; cellular half life; redox state; and structural conformation, etc.; and age-associated parameters, i.e., characteristics of young or old cells or organisms such as stress resistance, lifespan, doubling time, telomere length, physiological characteristics, appearance, disease states, etc. "Functional effects" include *in vitro*, *in vivo*, and *ex vivo* activities. The

functional effect can be measured in a host cell, organelle (e.g., isolated mitochondria), host cell membrane, isolated organelle membrane (e.g., isolated mitochondrial membrane), cellular extract, organelle extract (e.g., mitochondrial extract) or host organism.

By "determining the functional effect" is meant assaying for a compound that increases or decreases a parameter that is indirectly or directly under the influence of aging associated proteins or genes, e.g., measuring physical and chemical or phenotypic effects. Such functional effects can be measured by any means known to those skilled in the art, e.g., changes in spectroscopic characteristics (e.g., fluorescence, absorbance, refractive index); hydrodynamic (e.g., shape); chromatographic; or solubility properties for the protein; measuring inducible markers or transcriptional activation of the protein; measuring binding activity or binding assays, e.g. binding to antibodies; measuring changes in ligand binding activity; measuring cellular proliferation or lifespan; measuring cell surface marker expression; measurement of changes in protein levels for associated sequences; measurement of RNA stability; phosphorylation or dephosphorylation; signal transduction, e.g., receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP₃, or intracellular Ca²⁺); identification of downstream or reporter gene expression (CAT, luciferase, β-gal, GFP and the like), e.g., via chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, and ligand binding assays.

"Ligand" refers to a molecule that is specifically bound by a protein.

"Substrate" refers to a molecule that binds to an enzyme and is part of a specific chemical reaction catalyzed by the enzyme.

"Cofactor" refers to an additional component required for activity of an enzyme. (Leninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). A cofactor may be inorganic such as Fe, Cu, K, Ni, Mo, Se, Zn, Mn or Mg ions, or an organic molecule also known as a coenzyme. Coenzymes include flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), heme, coenzyme A, pyrodoxal phosphate, thiamine pyrophosphate, 5'-deoxyadenosylcobalamin, biocytin, tetrahydrofolate, retinal, and 1,25-dihydroxycholecalciferol. A co-factor can also include a protein subunit bound to the co-factor.

"Inhibitors", "activators", and "modulators" of aging associated genes and proteins are used to refer to activating, inhibitory, or modulating molecules identified using *in vitro* and *in vivo* assays of aging associated proteins and genes. Inhibitors are compounds that, e.g., bind to, partially or totally block activity, decrease, prevent, delay activation,

inactivate, desensitize, or down regulate the activity or expression of aging associated proteins and genes, e.g., antagonists. "Activators" are compounds that increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up regulate aging associated proteins. Inhibitors, activators, or modulators also include genetically modified versions of aging associated proteins and genes, e.g., versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, antibodies, antisense molecules, ribozymes, small chemical molecules and the like. Such assays for inhibitors and activators include, e.g., expressing aging associated proteins *in vitro*, in cells, or cell membranes, applying putative modulator compounds, and then determining the functional effects on activity, as described above.

Samples or assays comprising aging associated proteins and genes that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative protein or gene activity value of 100%. Inhibition of aging associated proteins or genes is achieved when the activity value relative to the control is about 80%, preferably 50%, more preferably 25-0%. Activation of aging associated proteins or genes is achieved when the activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

The term "test compound" or "drug candidate" or "modulator" or grammatical equivalents as used herein describes any molecule, either naturally occurring or synthetic, e.g., protein, oligopeptide (e.g., from about 5 to about 25 amino acids in length, preferably from about 10 to 20 or 12 to 18 amino acids in length, preferably 12, 15, or 18 amino acids in length), small organic molecule, polysaccharide, lipid, fatty acid, polynucleotide, oligonucleotide, etc., to be tested for the capacity to directly or indirectly modulation lymphocyte activation. The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a sufficient range of diversity. Test compounds are optionally linked to a fusion partner, e.g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a "lead compound") with some desirable property or activity, e.g., inhibiting activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

A "small organic molecule" refers to an organic molecule, either naturally occurring or synthetic, that has a molecular weight of more than about 50 daltons and less than about 2500 daltons, preferably less than about 2000 daltons, preferably between about 100 to about 1000 daltons, more preferably between about 200 to about 500 daltons.

5 "Biological sample" include sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes. Such samples include blood, sputum, tissue, cultured cells, e.g., primary cultures, explants, and transformed cells, stool, urine, etc. A biological sample is typically obtained from a eukaryotic organism, e.g., *C. elegans*, most preferably a mammal such as a primate e.g., chimpanzee or human; cow; dog; 10 cat; a rodent, e.g., guinea pig, rat, mouse; or a rabbit.

The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 50% identity, preferably 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 15 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region (e.g., the *C. elegans* proteins provided herein), when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection. Such sequences are then said to be 20 "substantially identical." This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 25 50-100 amino acids or nucleotides in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default 30 program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20

to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison
5 can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms
10 (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which
15 are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first
20 identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits
25 are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when:
30 the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation

(E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both strands.

5 "Nucleic acid" refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

10 Unless otherwise indicated, a particular nucleic acid sequence also implicitly encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

15 A particular nucleic acid sequence also implicitly encompasses "splice variants." Similarly, a particular protein encoded by a nucleic acid implicitly encompasses any protein encoded by a splice variant of that nucleic acid. "Splice variants," as the name suggests, are products of alternative splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternate) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternate splicing of exons. Alternate polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this definition.

20 The terms "polypeptide," "peptide" and "protein" are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers

in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "amino acid" refers to naturally occurring and synthetic amino acids, 5 as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is 10 bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical 15 compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to essentially identical sequences. Because of the degeneracy of the genetic code, a large 20 number of functionally identical nucleic acids encode any given protein. For instance, the codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified 25 variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a 30

polypeptide is implicit in each described sequence with respect to the expression product, but not with respect to actual probe sequences.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid.

Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (*see, e.g., Creighton, Proteins* (1984)).

Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, *see, e.g., Alberts et al., Molecular Biology of the Cell* (3rd ed., 1994) and Cantor and Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains, e.g., extracellular domains, transmembrane domains, and cytoplasmic domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 15 to 350 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of β -sheet and α -helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units. Anisotropic terms are also known as energy terms.

A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include ^{32}P , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins

which can be made detectable, e.g., by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at

65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing 5 temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, e.g., in Innis *et al.* (1990) *PCR* 10 *Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.).

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize 15 under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional 20 guidelines for determining hybridization parameters are provided in numerous reference, e.g., and Current Protocols in Molecular Biology, ed. Ausubel, *et al*

"Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, 25 epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

30 An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily

responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, 5 pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab')_2$, a dimer of Fab which itself is a light chain joined to $V_H\text{-}C_{H1}$ by a disulfide bond. The $F(ab')_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab')_2$ dimer into an Fab' monomer. The Fab' monomer is essentially Fab with part of the hinge region (see *Fundamental Immunology* (Paul ed., 3d ed. 10 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole antibodies, or those synthesized *de novo* using recombinant DNA methodologies (e.g., single 15 chain Fv) or those identified using phage display libraries (see, e.g., McCafferty *et al.*, *Nature* 348:552-554 (1990))

For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many technique known in the art can be used (see, e.g., Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor *et al.*, *Immunology Today* 4: 72 (1983); Cole *et al.*, pp. 20 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or 25 other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty *et al.*, *Nature* 348:552-554 (1990); Marks *et al.*, *Biotechnology* 10:779-783 (1992)).

A "chimeric antibody" is an antibody molecule in which (a) the constant 30 region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable

region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

In one embodiment, the antibody is conjugated to an "effector" moiety. The effector moiety can be any number of molecules, including labeling moieties such as 5 radioactive labels or fluorescent labels, or can be a therapeutic moiety. In one aspect the antibody modulates the activity of the protein.

The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein, often in a heterogeneous 10 population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and more typically more than 10 to 100 times background. Specific binding to an antibody under such conditions requires an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to aging associated proteins, polymorphic variants, 15 alleles, orthologs, and conservatively modified variants, or splice variants, or portions thereof, can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with aging associated proteins and not with other proteins. This selection may be achieved by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically 20 immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, *Antibodies, A Laboratory Manual* (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

25 ASSAYS FOR GENES AND GENE PRODUCTS THAT REGULATE AGING

Genetic and other models can be used to identify mutants, phenotypes (mediated by mutants and by RNAi), genes, and gene products in the aging process, e.g., 30 RNAi analysis; microarray analysis; chemical mutagenesis; mammalian complementation assays for age-associated proteins; yeast two hybrid assays, immunoprecipitation; alteration in age-associated reporter gene expression or localization (e.g., daf-2 or daf-16); overexpression, underexpression, or knockout of gene expression, etc. Suitable controls include organisms with altered lifespan, e.g., by mutation or RNAi. These assays can be used with eukaryotic organisms, cells, and organelles such as mitochondria. The genes and gene products associated with a mutation are then identified and used to analyze the aging process

at a molecular level. Genes and gene products that regulate the aging process can be identified under normal aging conditions. Patterns of gene expression that correlate with normal or abnormal aging can also be used to identify genes associated with aging. The aging process has likely been conserved throughout evolution. Thus, genes and gene
5 products that regulate the aging process in one species will be useful to identify similar or orthologous genes and gene products in divergent species.

A. *Manifestations of the Aging Process*

The most obvious disruption of the aging process is a change in lifespan of an
10 individual. Lifespan can either be increased or decreased by a mutation in a gene that participates in the aging process or, as shown here, by another intervention, e.g., RNAi mediated silencing of such a gene. In addition, for all eukaryotic organisms other physical characteristics can be used to distinguish young individuals from older individuals. Thus, at an organismal level, a mutation that affects the aging process will usually affect the lifespan
15 of an individual and may also affect other aging characteristics of that individual. Such manifestations of the aging process are known as "age-associated parameters," e.g., indicia from Nomarski analysis, stress resistance, appearance, physiological changes, disease states, loss of doubling capacity, changes in differentiated phenotype, indirect effects such as fusion protein expression and localization or posttranscriptional modification, etc., are described in
20 more detail below.

Those of skill in the art will recognize that the aging process can also be manifested at an organismal level or at a cellular level. While a list of characteristics of aging is provided below, it is not exhaustive and other characteristics of the aging process may also be analyzed within the scope of the present invention.

Characteristics of aging can be distinguished at the organismal level and may
25 be species specific. For example, characteristics of older human individuals include skin wrinkling, graying of the hair, baldness, cataracts, hypermelanosis, osteoporosis, cerebral cortical atrophy, lymphoid depletion, thymic atrophy, increased incidence of diabetes type II, atherosclerosis, cancer, and heart disease (Nehlin *et al.*, *Annals NY Acad. Sci.*, 980:176-179
30 (2000)). Other characteristics of mammalian aging include the following: weight loss; lordokyphosis (hunchback spine); absence of vigor; lymphoid atrophy; decreases in bone density, dermal thickness, and subcutaneous adipose tissue; decreased ability to tolerate stresses, such as wound healing, anesthesia, and response to hematopoietic precursor cell

ablation; sparse hair; liver pathologies; atrophy of intestinal villi; skin ulceration; amyloid deposits; and joint diseases (Tyner *et al.*, *Nature* 415:45-53 (2002)).

Careful observation reveals characteristics of the aging process in other eukaryotes, including invertebrates. For example, characteristics of aging in the model 5 nematode *C. elegans* as observed by Nomarski analysis include slow movement, flaccidity, yolk accumulation, intestinal autofluorescence (lipofuscin), loss of ability to chew and expel (distended oral and anal cavities), necrotic cavities in tissue, curdled appearing tissue, and germ cell appearance (graininess, large, well separated nuclei, fewer nuclei, and cavities).

Characteristics of aging can also be observed in cultured cells and also in 10 mitochondria. Note that many of these characteristics can also be observed in animals. Normal eukaryotic cells have a defined lifespan when taken from the organism grown in culture. These "primary" tissue culture cells are cells that have neither been immortalized nor acquired a transformed phenotype. The primary cells will divide a defined number of times in culture and then die (reviewed in Campisi, *Exper. Geron.* 36:6-7-618 (2001)).

15 Cellular aging is also characterized by changes other than loss of doubling capability, e.g. changes in apoptotic death and changes in differentiated phenotypes (*Id.*). In some cases, cellular characteristics of aging can also be observed in immortalized or transformed cell lines. Aging cells also show stress resistance, e.g., free radical generation and H₂O₂ resistance. Age-related bio-markers, gene, and protein expression patterns may also be used 20 to determine or measure aging.

Finally, aging can be assessed indirectly, by an aging related functional effects (phenotypic, physical, and chemical effects), e.g., gene expression (e.g., transcript abundance), protein abundance/localization/modification state, chromatin structure, signal transduction, second messenger levels, marker expression, phosphorylation, 25 posttranscriptional modification, reporter gene expression, reporter or fusion protein localization, etc. Such effects can often be monitored when examining upstream or downstream genetic or biochemical pathways of an aging associated gene. Such effects can also be monitored using the aging associated gene.

In one embodiment, a test compound is contacted to one or more cells of an 30 organism or one or more culture cells, and the one or more cells, or the entire organism is evaluated. In particular, a characteristic of aging (e.g., a direct observation or an aging-related functional effect) can be evaluated to determine the test compound has an affect on aging or an aging-related process such as stress resistance or metabolism.

B. *Isolation of Genes Associated with Aging*

Those of skill in the art will recognize that aging associated nucleic acids and proteins may be conserved in divergent species. Thus, the sequence of a nucleic acid or protein associated with aging in one species can be used to identify aging associated nucleic acids and proteins from other species, as well as genetic and biochemical pathways for the aging associated genes. For example, using methods described in this specification, aging associated genes identified in *C. elegans* can be used to identify aging associated genes or proteins in humans or other higher eukaryotes.

10 Isolation of genes and gene products associated with aging using classical genetic methods.

Using classical genetic methods (random genomic mutagenesis), aging mutants are generated by mutagenesis. The mutagenesis protocol will depend on the organism. For example, some eukaryotic organisms can be randomly mutagenized chemically by treatment with compounds like ethane methyl sulfonate (EMS) or can be mutagenized by exposure to UV or gamma irradiation. Preferably, these compounds would be used on organisms such as mammalian cells, yeast, *C. elegans*, *Drosophila melanogaster*, or zebrafish.

Mutants in the aging process will preferably be characterized by an increase or 20 a decrease in lifespan. Mutants in the aging process will also preferably exhibit a temporal change in expression of an aging characteristic, including those listed above. Those of skill in the art will recognize that mutants can be generated in many ways depending on the organism and phenotypes studied. Typically, the mutagenesis process decreases, increases, or changes gene activity. Examples of such mutants include age-1, daf-2, and daf-16 in *C. elegans*.

Isolation of genes and gene products associated with aging using gene inactivation.

In another embodiment, aging mutants are made by inactivation of a gene of interest, using methods other than classical genetic mutagenesis methods. The gene of interest can be inactivated, e.g., using dsRNA inhibition, by using antisense technology, or can be inactivated by homologous recombination. The inactivation can take place in a multicellular organism or in cultured cells. For example, the p66 gene has been removed from mice using homologous recombination, creating a mouse with a longer lifespan than

wildtype. Transgenic mice of interest which show lifespan increase include Ames dwarf mutant mice, p66(-/-) knockout mice, alpha MUPA and MGMT transgenic mice (see, e.g., Anisimov, *Mech Aging Dev.* 122:1221-1255 (2001); Lithgow & Andersen, *Bioessays* 22:410-413 (2000)).

5 dsRNA inhibition can also be used to screen a large number of genes for a phenotype. The method is preferably done in an organism whose genome has been sequenced. DNA fragments corresponding to predicted genes are cloned into a vector between two bacterial promoters in inverted orientation. The library is then transformed into a bacterial strain capable of expressing the DNA fragments. The transformed bacteria or the
10 library DNA alone is then introduced into the experimental organism. If desired, inducible promoters can be used and expression of the inhibitory dsRNA can be induced during a particular time of development or under desired conditions.

15 A preferred embodiment uses a library whose members each include a DNA fragment from *C. elegans*. Each library member is transformed into *E. coli* and the *E. coli* fed to the worms. The DNA fragments are under the control of T7 promoters. The bacteria express a T7 polymerase that is inducible by IPTG, rendering expression of the inhibitory dsRNA inducible by IPTG.

20 Isolation of genes and gene products associated with aging using overexpression.

In another embodiment, aging mutants are made by overexpressing a gene associated with aging, using methods other than classical genetic mutagenesis methods.. The gene associated with aging is cloned into a vector under the control of a promoter appropriate for the experimental system. The expression vector is then introduced into the experimental system. The overexpression can take place in either a multicellular organism or in cultured cells.

25 Isolation of genes and gene products associated with aging using naturally occurring mutants.

30 Aging mutants can also occur naturally. Those of skill in the art will recognize that such mutants do exist and can be used in the present invention. For example, in humans, several premature aging syndromes have been characterized including Werner syndrome, Hutchinson-Guilford disease, Bloom's syndrome, Cockayne's syndrome, ataxia

telangiectasia, and Down's syndrome. Where appropriate, cells from an individual afflicted with an aging syndrome can be studied, rather than the whole organism.

5 Isolation of genes and gene products associated with aging using genetic or biochemical pathways known to regulate aging.

Genetic analysis can also be used to delineate regulatory pathways and determine functional relationships between genes and gene products. In the case of a complex biological process such as aging, more than one regulatory pathway may regulate the aging process. Those of skill in the art will recognize that genetic analysis of mutants can 10 be used to characterize regulatory pathways and determine relationships between genes. Of course, it is also possible to use RNA interference to modulate gene activity in analyzing the regulatory pathways and relationships.

An example of genetic analysis of a regulatory pathway is found in *C. elegans*. The *daf-2* gene encodes an insulin/IGF-1 receptor homologue. Mutations that lower the level 15 of *daf-2* result in animals that have enhanced lifespans. (For review see Guarente and Kenyon, *Nature* 408:255-262 (2000)). *daf-16* encodes a forkhead transcription factor homologue that acts downstream of *daf-2* and is required for *daf-2* activity. *daf-16* mutants have short lifespans. Newly isolated mutations can be analyzed for interaction with the *daf2/daf16* pathway. In that way, genes and gene products can be assigned to a regulatory 20 pathway.

In addition, genes that interact with the pathway can be identified by using an appropriate mutant screen. For example, the *C. elegans* protein DAF-16 is a transcriptional activator. A fusion protein between DAF-16 and green fluorescent protein (DAF-16/GFP) can be used to identify the cellular location of the protein. In wild-type animals the protein is 25 localized throughout cells. In long-lived *daf-2* mutants, DAF-16 is localized to the nucleus.

Those of skill in the art will recognize that the localization of DAF-16/GFP can be used to identify mutants that perturb the *daf2/daf16* pathway. Localization of DAF-16/GFP to the nucleus can be used to screen for drugs that enhance lifespan or mutations that enhance lifespan.

30 A similar fusion using an end product of the pathway, superoxide dismutase (SOD-3), was constructed. Long lived mutants of the *daf-2/daf-16* pathway expressed SOD-3/GFP at a higher level than wild-type worms. Levels of fluorescence from SOD-3/GFP can be followed by microscopy. Those of skill in the art will recognize that expression of SOD-3/GFP can be used to screen for long-lived mutants.

Isolation of genes and gene products associated with aging using changes in expression levels.

Those of skill in the art will recognize that levels of messenger RNA can be measured during the aging process. For aging associated proteins, changes in mRNA levels can be detected either during normal aging process or when comparing an aging mutant to a wild-type individual. Changes in mRNA levels can be measured using techniques known to those of skill in the art, including microarrays, northern blots, and RT PCR.

Aging associated genes can be identified through the use of microarrays where changes in expression of mRNA levels under different conditions or at different times of development can be assayed (see Tables 5 and 6). mRNA levels can also be analyzed in aging mutants to identify genes that are affected by increases or decreases in lifespan.

Microarrays are made by methods known to those of skill in the art, or are purchased. Gene expression profiles for the genes described herein can be generated and used for comparison to identify other age-associated genes. The profile can be generated using a microarray, or by other means. The profiles can be derived from animals, cells, mitochondria, or other suitable sources expressing the genes of interest, e.g., RNAi treated cells or animals. Such profiles can be stored as computer files and analyzed or compared to identify additional genes using algorithms known to those of skill in the art.

Moreover, a gene identified by any method, e.g., transcript or protein profiling, RNAi, or genetic mutation, can then be analyzed by one of the other methods. For example, the activity of a gene whose transcription is correlated with aging can be altered using RNAi. Further, chromosomal deficiencies and genetic mutations can be identified in the gene of interest. These exemplary alterations can be used to evaluate the contribution of a gene or gene product to the aging phenotype. The functional relevance of genes so identified can be tested with mutants or RNAi.

COMPUTER ASSISTED DRUG DESIGN AND PROFILING

Yet another assay for compounds that modulate aging involves computer assisted drug design, in which a computer system is used to generate a three-dimensional structure of an aging associated protein based on the structural information encoded by the amino acid or nucleic acid sequence. The input amino acid sequence interacts directly and actively with a pre-established algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then

examined to identify regions of the structure that have the ability to bind, e.g., ligands, substrates, cofactors, etc. These regions are then used to identify ligands that bind to the protein.

The three-dimensional structural model of the protein is generated by entering
5 an aging associated protein amino acid sequences of at least 25, 50, 75 or 100 amino acid residues or corresponding nucleic acid sequences encoding an aging associated protein into the computer system. The amino acid sequence represents the primary sequence or subsequence of each of the proteins, which encodes the structural information of the protein. At least 25, 50, 75, or 100 residues of the amino acid sequence (or a nucleotide sequence
10 encoding at least about 25, 50, 75 or 100 amino acids) are entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (e.g., magnetic diskettes, tapes, cartridges, and chips), optical media (e.g., CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of the aging-associated protein is then generated by the
15 interaction of the amino acid sequence and the computer system, using software known to those of skill in the art. The resulting three-dimensional computer model can then be saved on a computer readable substrate. For example, three-dimensional models of the structures of a number of proteins described here are known and can be used to model homologs and interactions with other chemical compounds. See, e.g., Damberger *et al.* *Protein Sci.* 1994
20 Oct;3(10):1806-21. (HSF structure); Harrison *et al.* *Science.* 1994 Jan 14;263(5144):224-7 (HSF structure); Lange *et al.* *Proc Natl Acad Sci U S A.* 2002 Mar 5;99(5):2800-5 (cytochrome bc₁ complex); Iwata, *et al.*, *Science.* 1998 Jul 3;281(5373):64-71; Gibbons *et al.*, *Nat Struct Biol.* 2000 Nov;7(11):1055-61 (F(1)-ATPase); Faig *et al.*, (2001) *Structure* (Camb). 2001 Aug;9(8):659-67 (NAD(P)H:quinone oxidoreductase 1); Ingelman *et al.*
25 *Biochemistry.* 1999 Jun 1;38(22):7040-9. (NAD(P)H:flavin oxidoreductase).

The amino acid sequence represents a primary structure that encodes the information necessary to form the secondary, tertiary and quaternary structure of the aging associated protein. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as "energy terms," or anisotropic terms and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program is therefore using these terms

encoded by the primary structure or amino acid sequence to create the secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can 5 enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, e.g., cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary 10 structure with like.

Once the structure has been generated, potential ligand and substrate binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is 15 then compared to that of the aging associated protein to identify ligands that bind to the aging associated protein, orthologs thereof, etc. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein.

Computer systems are also used to screen for mutations, polymorphic variants, 20 alleles and interspecies homologs of the aging associated protein or gene. Such mutations can be associated with disease states. Once the variants are identified, diagnostic assays can be used to identify patients having such mutated genes associated with disease states. Identification of the mutated aging associated protein involves receiving input of a first 25 nucleic acid, e.g., SEQ ID NOS:1-11, and genes disclosed in Tables 5 and 6 and orthologs or conservatively modified versions thereof. The sequence is entered into the computer system as described above. The first nucleic acid or amino acid sequence is then compared to a second nucleic acid or amino acid sequence that has substantial identity to the first sequence. The second sequence is entered into the computer system in the manner described above. Once the first and second sequences are compared, nucleotide or amino acid differences 30 between the sequences are identified. Such sequences can represent allelic differences in aging associated protein, e.g., human genes and mutations associated with disease states. The first and second sequences described above can be saved on a computer readable substrate.

Nucleic acids encoding aging associated proteins can be used with high density oligonucleotide array technology (e.g., GeneChipTM) to identify family members and

homologs, orthologs, alleles, conservatively modified variants, and polymorphic variants in this invention. In the case where the homologs being identified are linked to a known disease, they can be used with GeneChip™ as a diagnostic tool in detecting the disease in a biological sample, *see, e.g.*, Gunthand *et al.*, *AIDS Res. Hum. Retroviruses* 14: 869-876 5 (1998); Kozal *et al.*, *Nat. Med.* 2:753-759 (1996); Matson *et al.*, *Anal. Biochem.* 224:110-106 (1995); Lockhart *et al.*, *Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al.*, *Genome Res.* 8:435-448 (1998); Hacia *et al.*, *Nucleic Acids Res.* 26:3865-3866 (1998).

In another aspect, the invention features a computer medium having a plurality of digitally encoded data records. Each data record includes a value representing the level of expression of one or more age-associated gene as described herein in a sample, and a descriptor of the sample. The level of expression can relate to mRNA level and/or protein levels. The record can further include an aging-associated parameter as described herein. The descriptor of the sample can indicate a subject from which the sample was derived (e.g., a patient, a mutant animal), a treatment (e.g., RNAi treatment), or a location of the 10 sample. In one embodiment, the data record further includes values representing the level of expression of genes and proteins other than an age-associated gene of the invention (e.g., other genes associated with an aging-disorder, or other genes on an array). The data record can be structured as a table, e.g., a table that is part of a database such as a relational database 15 (e.g., a SQL database of the Oracle or Sybase database environments).

Also featured is a method of evaluating a sample. The method includes providing a sample, e.g., from the subject, and determining an expression profile of the sample, wherein the profile includes a value representing the level expression of an age-associated gene described herein. The method can further include comparing the value or the profile (i.e., multiple values) to a reference value or reference profile. The gene 20 expression profile of the sample can be obtained by any of the methods described herein (e.g., by providing a nucleic acid from the sample and contacting the nucleic acid to an array). The method can be used to infer a longevity-associated phenotype in a subject wherein an increase or decrease expression of an age-associated gene described herein is an indication 25 that the subject has or is disposed to having an altered longevity-associated phenotype. The method can be used to monitor a treatment for an aging in a subject. For example, the gene expression profile can be determined for a sample from a subject undergoing treatment. The profile can be compared to a reference profile or to a profile obtained from the subject prior 30 to treatment or prior to onset of the disorder (*see, e.g.*, Golub *et al.*, *Science* 286:531 (1999)).

ISOLATION OF NUCLEIC ACIDS ENCODING AGING ASSOCIATED PROTEINS

This invention can include use of routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, 5 *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

Aging associated protein-encoding nucleic acids, polymorphic variants, orthologs, and alleles can be isolated using the *C. elegans* genes provided herein using, e.g., moderate or low stringent hybridization conditions, by screening libraries, by analyzing a 10 sequence database, and/or by synthetic gene construction. Alternatively, expression libraries can be used to clone aging associated proteins, polymorphic variants, orthologs, and alleles by detecting expressed homologs immunologically with antisera or purified antibodies made against *C. elegans* or mammalian aging associated proteins or portions thereof or by complementation, e.g., of a *C. elegans* phenotype. In a preferred embodiment, human nucleic 15 acid libraries are screened for homologues of *C. elegans* genes or proteins that are associated with aging.

To make a cDNA library, one can choose a source that is rich in the RNA of choice. The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfixed into a recombinant host for propagation, screening and 20 cloning. Methods for making and screening cDNA libraries are well known (*see, e.g.*, Gubler & Hoffman, *Gene* 25:263-269 (1983); Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are 25 constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

An alternative method of isolating aging associated protein-encoding nucleic 30 acid and their orthologs, alleles, mutants, polymorphic variants, and conservatively modified variants combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (*see U.S. Patents 4,683,195 and 4,683,202; PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid

sequences of aging associated protein-encoding genes directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify homologs using the sequences provided herein. Restriction endonuclease sites can be incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of aging associated protein encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate vector.

Gene expression of aging associated proteins can also be analyzed by techniques known in the art, e.g., reverse transcription and amplification of mRNA, isolation of total RNA or poly A⁺ RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, high density polynucleotide array technology, e.g., and the like.

Nucleic acids encoding aging associated proteins can be used with high density oligonucleotide array technology (e.g., GeneChipTM) to identify aging associated proteins, orthologs, alleles, conservatively modified variants, and polymorphic variants in this invention. In the case where the homologs being identified are linked to modulation of aging associated proteins, they can be used with GeneChipTM as a diagnostic tool in detecting the disease in a biological sample, see, e.g., Gunthand *et al.*, *AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al.*, *Nat. Med.* 2:753-759 (1996); Matson *et al.*, *Anal. Biochem.* 224:110-106 (1995); Lockhart *et al.*, *Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al.*, *Genome Res.* 8:435-448 (1998); Hacia *et al.*, *Nucleic Acids Res.* 26:3865-3866 (1998).

The gene for aging associated proteins are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, e.g., plasmids, or shuttle vectors.

EXPRESSION IN PROKARYOTES AND EUKARYOTES

To obtain high level expression of a cloned gene, such as those cDNAs encoding aging associated proteins, one typically subclones aging associated protein encoding nucleic acids into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, e.g., in Sambrook *et al.*, and Ausubel *et al.*, *supra*. Bacterial expression systems for

expressing aging associated proteins are available in, e.g., *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva *et al.*, *Gene* 22:229-235 (1983); Mosbach *et al.*, *Nature* 302:543-545 (1983). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also 5 commercially available.

Selection of the promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can 10 be accommodated without loss of promoter function.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of aging associated protein encoding nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence 15 encoding aging associated proteins and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

In addition to a promoter sequence, the expression cassette should also contain 20 a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in 25 eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as MBP, GST, and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc.

Expression vectors containing regulatory elements from eukaryotic viruses are 30 typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus

promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Expression of proteins from eukaryotic vectors can be also be regulated using inducible promoters. With inducible promoters, expression levels are tied to the concentration of inducing agents, such as tetracycline or ecdysone, by the incorporation of response elements for these agents into the promoter. Generally, high level expression is obtained from inducible promoters only in the presence of the inducing agent; basal expression levels are minimal. Inducible expression vectors are often chosen if expression of the protein of interest is detrimental to eukaryotic cells.

Some expression systems have markers that provide gene amplification such as thymidine kinase and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with mitochondrial respiratory chain protein encoding sequences and glycolysis protein encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of aging associated proteins, which are then purified using standard techniques (see, e.g., Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983)).

Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, biolistics, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material

into a host cell (*see, e.g.*, Sambrook *et al.*, *supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing aging associated proteins.

After the expression vector is introduced into the cells, the transfected cells are 5 cultured under conditions favoring expression of aging associated proteins, which is recovered from the culture using standard techniques identified below.

Expression vectors with appropriate regulatory sequences can also be used to express a heterologous gene in a nematode. In one example, the expression vector is injected 10 in the gonad of the nematode, and the vector is incorporated, *e.g.*, as an extra-chromosomal array in progeny of the nematode. The vector can further include a second gene (*e.g.*, a marker gene) that indicates the presence of the vector. For example, the heterologous gene can be a mammalian gene, *e.g.*, a mammalian cDNA, or a fragment thereof.

PURIFICATION OF AGING ASSOCIATED PROTEINS

15 Either naturally occurring or recombinant aging associated proteins can be purified for use in functional assays. Naturally occurring aging associated proteins can be purified, *e.g.*, from human tissue. Recombinant aging associated proteins can be purified from any suitable expression system.

Aging associated proteins may be purified to substantial purity by standard 20 techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.*, Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al.*, *supra*; and Sambrook *et al.*, *supra*).

A number of procedures can be employed when recombinant aging associated 25 proteins are being purified. For example, proteins having established molecular adhesion properties can be reversibly fused to aging associated proteins. With the appropriate ligand, aging associated proteins can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, aging associated proteins could be purified using immunoaffinity columns.

30

A. *Purification of aging associated proteins from recombinant bacteria*

Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction

with IPTG is one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein.

Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of aging associated protein inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, e.g., by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French Press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (see, e.g., Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein. Other suitable buffers are known to those skilled in the art. Aging associated proteins are separated from other bacterial proteins by standard separation techniques, e.g., with Ni-NTA agarose resin.

Alternatively, it is possible to purify aging associated proteins from bacteria periplasm. After lysis of the bacteria, when the aging associated proteins are exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO₄ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted

and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

B. Standard protein separation techniques for purifying aging associated
5 proteins

Solubility fractionation

Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 10 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of 15 proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

20

Size differential filtration

The molecular weight of the aging associated proteins can be used to isolate it 25 from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of 30 the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

Column chromatography

5 The aging associated proteins can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

IMMUNOLOGICAL DETECTION OF AGING ASSOCIATED PROTEINS

10 In addition to the detection of aging associated genes and gene expression using nucleic acid hybridization technology, one can also use immunoassays to detect aging associated proteins of the invention. Such assays are useful for screening for modulators of aging associated proteins, e.g., for regulation of lifespan, as well as for therapeutic and diagnostic applications. Immunoassays can be used to qualitatively or quantitatively analyze 15 aging associated proteins. A general overview of the applicable technology can be found in Harlow & Lane, *Antibodies: A Laboratory Manual* (1988).

20 Methods of producing polyclonal and monoclonal antibodies that react specifically with the aging associated proteins are known to those of skill in the art (see, e.g., Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include antibody preparation by selection of 25 antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (see, e.g., Huse *et al.*, *Science* 246:1275-1281 (1989); Ward *et al.*, *Nature* 341:544-546 (1989)).

30 A number of immunogens comprising portions of aging associated proteins may be used to produce antibodies specifically reactive with an aging associated protein. For example, recombinant protein or an antigenic fragment thereof, can be isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing

antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (e.g., BALB/C mice) or rabbits is immunized with the 5 protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the beta subunits. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for 10 antibodies reactive to the protein can be done if desired (*see, Harlow & Lane, supra*).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (*see, Kohler & Milstein, Eur. J. Immunol. 6:511-519 (1976)*). Alternative methods of immortalization include 15 transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may 20 isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse, *et al.*, *Science 246:1275-1281 (1989)*.

Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the 25 immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of 10^4 or greater are selected and tested for their cross reactivity against non-specific proteins, using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with a K_d of at least about 0.1 mM, more usually at least about 1 μM , preferably at least about 0.1 μM or better, and most preferably, 0.01 μM or better. Antibodies 30 specific only for a particular ortholog, such as a human ortholog, can also be made, by subtracting out other cross-reacting orthologs from a species such as a non-human mammal.

Once the specific antibodies against aging associated proteins are available, the protein can be detected by a variety of immunoassay methods. In addition, the antibody can be used therapeutically as aging associated protein modulators, e.g., to enhance and

extend lifespan or to prevent premature aging. For a review of immunological and immunoassay procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in Enzyme Immunoassay (Maggio, 5 ed., 1980); and Harlow & Lane, *supra*.

It is also possible to use protein arrays to detect an aging associated protein, e.g., to concurrently detect a plurality of aging associated proteins. Exemplary methods for producing protein arrays are provided in De Wildt *et al.* (2000) *Nat. Biotechnol.* 18:989-994; Lueking *et al.* (1999) *Anal. Biochem.* 270:103-111; Ge (2000) *Nucleic Acids Res.* 28, e3, I-VII; MacBeath and Schreiber (2000) *Science* 289:1760-1763; WO 0/98534, WO01/83827, 10 WO02/12893, WO 00/63701, WO 01/40803 and WO 99/51773. In some implementations, polypeptides (including peptides) are spotted onto discrete addresses of the array, e.g., at high speed, e.g., using commercially available robotic apparatus, e.g., from Genetic MicroSystems or BioRobotics. The array substrate can be, for example, nitrocellulose, plastic, glass, e.g., 15 surface-modified glass. The array can also include a porous matrix, e.g., acrylamide, agarose, or another polymer.

ASSAYS FOR MODULATORS OF AGING ASSOCIATED PROTEINS

A. Assays

Modulation of aging associated proteins and genes can be assessed using a variety of *in vitro* and *in vivo* assays, as described herein, and, such assays can be used to test for inhibitors and activators of aging associated proteins. Such modulators of aging 20 associated proteins and genes, which are involved in aging, are useful for enhancing lifespan or treating premature aging. Modulators of aging associated proteins and genes are tested using either recombinant or naturally occurring, preferably *C. elegans*, mouse, rat, guinea pig, 25 monkey, or human aging associated proteins.

An example of a modulator of the mitochondrial respiratory chain is antimycin. Antimycin inhibits the transfer of electrons from complex 2 to complex 3 during mitochondrial respiration. This drug has been found to extend the lifespan of *C. elegans*. Administration of antimycin to adult worms increased lifespan, while administration of 30 antimycin to worms from hatching through adulthood substantially increases lifespan. Mean lifespan of untreated worms was 17 days while animals grown in the presence of 0.1 micromolar antimycin in ethanol had a mean lifespan of 21 days.

Preferably, the aging associated proteins or genes will have a *C. elegans* or a mammalian, e.g., a rat, mouse, guinea pig, rabbit, monkey, or human sequence. Alternatively, the aging associated proteins or genes of the assay will be derived from a eukaryote and include an nucleic acid or amino acid subsequence having sequence identity to the *C. elegans* genes and gene products described herein. Generally, the sequence identity will be at least 30%, 35%, 40%, 45% or 50%, preferably at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90%, most preferably at least 95%.

Measurement of modulation of aging phenotype with aging associated proteins or cells expressing aging associated proteins or genes, either recombinant or naturally occurring, can be performed using a variety of assays, *in vitro*, *in vivo*, and *ex vivo*. A suitable physiological change that affects activity can be used to assess the influence of a test compound on the polypeptide or nucleic acid of this invention. When the functional effects are determined using intact cells or animals, one can also measure a variety of effects such as, increases or decreases in lifespan, cellular proliferation, or in the case of signal transduction, hormone release, transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as cGMP.

In a preferred embodiment, aging associated protein or gene modulators are assayed *in vivo* by screening in *C. elegans* or in a mammalian model system (cellular or animal) for changes in mean and median lifespan.

Some aging associated proteins have measurable enzymatic activity. Thus, enzymatic assays can be performed to identify compounds that modulate the enzymatic activity. Enzymatic activity can encompass a chemical reaction carried out by a protein, as well as binding of substrates, cofactors, regulatory compounds, or ligands to the protein. It may also be useful to monitor the affect of a test compound on other properties of the aging associated protein, e.g., a structural property (e.g., conformation, oligomerization state, stability, mobility, and the like) or a cellular property (e.g., cellular localization, accessibility, clustering, and the like).

The protein activity and binding capabilities assayed will depend on the aging associated protein. For example, the following proteins with known enzymatic activities have been associated with aging in *C. elegans*: cytochrome C₁ (gene C54G4.8, SEQ ID NO:1 Accession No. CAA99820.1), NADH oxidoreductase (gene T10E9.7, SEQ ID NO:2 Accession No. AAB522474.1), ATP synthase (gene F27C1.7, SEQ ID NO:3 Accession No. AAB37654.1), cytochrome C oxidase (gene F26E4.9, SEQ ID NO:4 Accession No.

CAB03002.1), phosphoglucose isomerase (gene Y87G2A.8, SEQ ID NO:5 Accession No. CAB60430.1), a GTPase (gene T23H2.5, SEQ ID NO:6 Accession No. AAC48200.1), and HSF-1 (gene Y53C10A.12, SEQ ID NO:11 Accession No. CAA22146), and the genes and gene products listed in Table 5 and Table 6

5 For activity of cytochrome C and C1, binding to the cofactor heme is important. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). Thus, changes in ability to bind heme may correlate with a mutant phenotype or change during aging. Cytochrome C1 is an electron-transferring protein that contains heme and is found in cytochrome reductase, a membrane-associated proton pump. Cytochrome C is a
10 water soluble protein that transfers electrons from cytochrome reductase to cytochrome oxidase. Changes in electron transport may also be seen during aging or as a result of an aging mutation. It is possible to monitor the redox state of the heme cofactor, for example, by spectroscopy.

NADH oxidoreductase is an oligomeric enzyme complex located in the inner
15 mitochondrial membrane. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). In *C. elegans* inhibition of the 30 Kd subunit resulted in enhanced lifespan. Thus, assays of NADH oxidoreductase activity could be associated with aging. It is possible to monitor the redox state of NADH, for example, by spectroscopy.

Cytochrome C oxidase catalyzes the transfer of electrons from cytochrome C to molecular oxygen. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). Those of skill in the art will recognize that this activity can be assayed spectrophotometrically. In addition to this activity, changes in binding to substrates cytochrome C and oxygen may also be assayed.

ATP synthase catalyzes the synthesis of ATP from ADP and orthophosphate.
25 (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). The energy of a proton gradient is used to release the ATP product from its binding site. The ATP synthase protein is a multisubunit enzyme. Inhibition of the *C. elegans* homologue of the delta subunit of ATP synthase resulted in an enhanced lifespan phenotype. Activity of the delta subunit is sensitive to the drug oligomycin. Stryer, page 546.

30 Phosphoglucose isomerase catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). The enzyme is involved in glycolysis in most higher organisms and in gluconeogenesis in mammals. An extracellular role has been reported for the protein as a nerve growth factor and cytokine. (Jeffery *et al.*, *Biochem.* 39:955-964 (2000)).

A GTPase enzyme catalyzes the hydrolysis of GTP to GDP. The reaction can be followed using radioactively labeled GTP an separating the reaction products from the reaction substrates.

HSF, a transcription factor, activates transcription by forming a tri-mer, 5 binding to a specific DNA sequence called a promoter and then recruiting RNA polymerases to begin transcription. Stress response and heat shock response proteins are known as chaperonins. Activation of transcription and binding to a target DNA site can measured *in vitro* using purified or partially purified components. Activation of transcription can also be measured *in vivo* using a reporter gene construct with these proteins.

10 The functional activities described above do not represent all of the enzymatic activities that could be found in aging associated proteins. For example, some aging proteins could act to down regulate transcription of messenger RNA. Still other aging proteins may be functional, e.g., as a structural scaffold or adaptor protein, e.g., they may or may not have an enzymatic activity.

15 Assays to identify compounds with modulating activity can be performed *in vitro*, e.g., in a test tube, or using isolated membranes, e.g., mitochondrial membranes, or using cellular or mitochondrial extracts. Exemplary assays can include, for example, methods described or referenced in Al-Awqati, *Annu. Rev. Cell Biol.* 2:179-199 (1986); Brand et al., *Biol. Rev. Cambridge Philosphic Soc.* 62:141-193 (1987); Capaldi et al., *FEBS Lett* 138:1-7 (1982); Casey, *Biochim. Biophys. Acta* 768:319-347 (1984); Erecinska et al., *J. Membr. Biol.* 70:1-14 (1982); Fillingame, *Annu. Rev. Biochem.* 49:1079-1113 (1980); Hamamoto, *Proc. Natl. Acad. Sci. USA* 82:2570-2573 (1985); Hatefi, *Annu. Rev. Biochem.* 54:1015-1070 (1985); Klingenberg, *Trends Biochem. Sci.* 4:249-252 (1979); LaNoue et al., *Annu. Rev. Biochem.* 48:871-922 (1979); Mitchell, *Nature* 191:144-148 (1961); Prince, 20 *Trends Biochem. Sci.* 13:159-160 (1988); Slater, *Trends Biochem. Sci.* 8:239-242 (1983); Srere, *Trends Biochem. Sci.* 7:375-378 (1982); Tzagoloff, *Mitochondria*, New York: Plenum 25 (1982); Weiss et al., *Biochem. Soc. Trans.* 15:100-102 (1987).

For example, the aging associated protein or gene is first contacted with a potential modulator and incubated for a suitable amount of time, e.g., from 0.5 to 48 hours. 30 In one embodiment, aging associated protein or gene expression levels are determined *in vitro* by measuring the level of protein or mRNA. The level of protein or nucleic acid is measured using immunoassays such as western blotting, ELISA and the like with an antibody that selectively binds to the polypeptide or a fragment thereof. For measurement of mRNA, amplification, e.g., using PCR, LCR, or hybridization assays, e.g., northern hybridization,

RNAse protection, dot blotting, are preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

5 Alternatively, a reporter gene system can be devised using an aging associated protein promoter operably linked to a reporter gene such as chloramphenicol acetyltransferase, firefly luciferase, bacterial luciferase, β -galactosidase and alkaline phosphatase. Furthermore, the gene or protein of interest can be used as an indirect reporter via attachment to a second reporter such as green fluorescent protein (*see, e.g.*, Mistili & 10 Spector, *Nature Biotechnology* 15:961-964 (1997)). The reporter construct is typically transfected into a cell. After treatment with a potential modulator, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

15 B. *Modulators*

The compounds tested as modulators of aging associated proteins and genes can be any small chemical compound, or a biological entity, such as a protein, e.g., an antibody, a sugar, a nucleic acid, e.g., an antisense oligonucleotide or a ribozyme, or a lipid. Alternatively, modulators can be genetically altered versions of an aging associated proteins 20 and genes. Typically, test compounds will be small chemical molecules and peptides, or antibodies, antisense molecules, or ribozymes. Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay 25 steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

30 In one preferred embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such "combinatorial chemical libraries" or "ligand libraries" are then screened in one or more assays, as described

herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical 5 compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the 10 number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating 15 chemical diversity libraries can also be used. Such chemistries include, but are not limited to: peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), 20 nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), 25 nucleic acid libraries (see Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (see, e.g., U.S. Patent 5,539,083), antibody libraries (see, e.g., Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (see, e.g., Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); 30 isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony,

Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, RU, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, 5 Columbia, MD, etc.).

In one embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the cell or tissue expressing aging associated proteins is attached to a solid phase substrate. In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators or ligands in a single day. In 10 particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 96 modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different compounds. It is possible to assay many 15 plates per day; assay screens for up to about 6,000, 20,000, 50,000, or 100,000 or more different compounds are possible using the integrated systems of the invention.

C. Solid State and soluble high throughput assays

In one embodiment the invention provides soluble assays using aging 20 associated proteins or genes, or a cell or tissue expressing aging associated proteins or genes, either naturally occurring or recombinant. In another embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the aging associated protein or gene is attached to a solid phase substrate.

In the high throughput assays of the invention, it is possible to screen up to 25 several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (e.g., 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- 30 about 1500 different compounds. It is possible to assay many plates per day; assay screens for up to about 6,000, 20,000, 50,000, or more than 100,000 different compounds are possible using the integrated systems of the invention.

The protein of interest, or a cell or membrane comprising the protein of interest can be bound to the solid state component, directly or indirectly, via covalent or non

covalent linkage e.g., via a tag. The tag can be any of a variety of components. In general, a molecule which binds the tag (a tag binder) is fixed to a solid support, and the tagged molecule of interest is attached to the solid support by interaction of the tag and the tag binder.

5 A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fc region of an immunoglobulin, etc.) Antibodies to molecules with natural binders such as biotin are also widely available and
10 appropriate tag binders; *see, SIGMA Immunochemicals 1998 catalogue SIGMA, St. Louis MO*). Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form a tag/tag binder pair.

15 Synthetic polymers, such as polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, and polyacetates can also form an appropriate tag or tag binder. Many other tag/tag binder pairs are also useful in assay systems described herein, as would be apparent to one of skill upon review of this disclosure.

20 Common linkers such as peptides, polyethers, and the like can also serve as tags, and include polypeptide sequences, such as poly gly sequences of between about 5 and 200 amino acids. Such flexible linkers are known to persons of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulfhydryl linkages, or heterofunctional linkages.

25 Tag binders are fixed to solid substrates using any of a variety of methods currently available. Solid substrates are commonly derivatized or functionalized by exposing all or a portion of the substrate to a chemical reagent which fixes a chemical group to the surface which is reactive with a portion of the tag binder. For example, groups which are suitable for attachment to a longer chain portion would include amines, hydroxyl, thiol, and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as glass surfaces. The construction of such solid phase biopolymer arrays is well described in the literature. *See, e.g., Merrifield, J. Am. Chem. Soc. 85:2149-2154 (1963) (describing solid phase synthesis of, e.g., peptides); Geysen et al., J. Immun. Meth. 102:259-274 (1987) (describing synthesis of solid phase components on pins); Frank & Doring, Tetrahedron 44:60316040 (1988) (describing synthesis of various peptide sequences*

on cellulose disks); Fodor *et al.*, *Science*, 251:767-777 (1991); Sheldon *et al.*, *Clinical Chemistry* 39(4):718-719 (1993); and Kozal *et al.*, *Nature Medicine* 2(7):753-759 (1996) (all describing arrays of biopolymers fixed to solid substrates). Non-chemical approaches for fixing tag binders to substrates include other common methods, such as heat, cross-linking by 5 UV radiation, and the like.

Another example of a high-throughput assay does not require immobilizing a target protein. Such examples include homogenous assays such as fluorescence resonance energy transfer and fluorescence polarization. Spectroscopy can also be used in a variety of ways. Assays can also be used to generate structure-activity relationships (SAR). A method 10 of analyzing an aging associated protein can also include assays that may not be traditionally associated with a particular throughput, e.g., certain NMR binding assays (e.g., SAR by NMR), calorimetry, crystallization, and so forth.

CELLULAR TRANSFECTION AND GENE THERAPY

15 The present invention provides the nucleic acids of aging associated proteins for the transfection of cells *in vitro* and *in vivo*. These nucleic acids can be inserted into any of a number of well-known vectors for the transfection of target cells and organisms as described below. The nucleic acids are transfected into cells, *ex vivo* or *in vivo*, through the interaction of the vector and the target cell. The nucleic acid, under the control of a promoter, 20 then expresses a protein of the present invention, thereby mitigating the effects of absent, partial inactivation, or abnormal expression of the gene of interest, or increasing lifespan in a subject with normal gene expression. For example, as described herein, overexpression of HSF-1 extends adult lifespan. The compositions are administered to a patient in an amount sufficient to elicit a therapeutic response in the patient. An amount adequate to accomplish 25 this is defined as "therapeutically effective dose or amount."

Such gene therapy procedures have been used to correct acquired and inherited genetic defects. The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies (for a review of gene therapy procedures, see 30 Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Mulligan, *Science* 926-932 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1998); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in

Current Topics in Microbiology and Immunology (Doerfler & Böhm eds., 1995); and Yu et al., *Gene Therapy* 1:13-26 (1994)).

PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION

5 Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., nucleic acid, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., *Remington's Pharmaceutical Sciences*, 17th ed., 1989). Administration can be in any convenient manner, e.g., by injection, oral 10 administration, inhalation, transdermal application, or rectal administration.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a 15 predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, 20 moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, e.g., sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

25 The compound of choice, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

Formulations suitable for parenteral administration, such as, for example, by 30 intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers,

and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of compounds can be presented in unit-dose or 5 multi-dose sealed containers, such as ampules and vials.

Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by nucleic acids for *ex vivo* therapy can also be administered intravenously or parenterally as described above.

The dose administered to a patient, in the context of the present invention 10 should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a 15 particular patient.

In determining the effective amount of the vector to be administered in the treatment or prophylaxis of conditions owing to diminished or aberrant expression of the protein of choice, the physician evaluates circulating plasma levels of the vector, vector toxicities, progression of the disease, and the production of anti-vector antibodies. In general, 20 the dose equivalent of a naked nucleic acid from a vector is from about 1 µg to 100 µg for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of therapeutic nucleic acid.

For administration, compounds and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced 25 cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

It is understood that the examples and embodiments described herein are for 30 illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example 1: Objective characteristics of aging identified using Nomarski differential interference contrast microscopy

A. *Introduction*

In this study, the tissues of wild-type animals were compared to those of long-lived and short-lived insulin/IGF-1 signaling mutants during the course of their lives. We have found that Nomarski differential interference contrast (DIC) microscopy provides an effective, rapid and convenient means of visualizing many features of tissue aging. Using this method, we have found that extensive tissue deterioration takes place during aging, not only in the post-mitotic somatic tissues of the animal, but also in a mitotic lineage, the germline. Our findings indicate that insulin/IGF-1 signaling influences lifespan by changing the rate at which the tissues age, and that this pathway governs the aging not only of the post-mitotic somatic cells, but mitotic lineages as well. Interestingly, the majority of animals we have examined whose adult lifespans were shortened by mutations or certain RNAi treatments did not look prematurely "old" upon observation with Nomarski microscopy. However, animals with lifespan mutations that caused shorter lifespans due to premature aging looked prematurely old (e.g., *hsp*). Thus, this technique aids in distinguishing mutated animals who die young, and mutated animals with premature aging, even though both classes of animals have shortened adult lifespans.

B. *Methods*

The following strains were used: N2, *daf-16(mu86)I*, *daf-2(e1370)III*, *daf-2(mu150)III*, *daf-2(e1370)III*; *deg-1(u38)X*, *ced-3(n1286)IV*, DH1033 *bIs1[vit-2::gfp; rol-6]; sqt-1(sc103)*, *daf-2(e1370)III*; *him-5(e1490)V*.

Lifespan Analysis: Wild-type, *daf-2* and *daf-16* animals raised at 20°C were shifted to 25°C at the L4 molt, and transferred to new plates every other day thereafter until progeny production ceased. Animals were judged to be dead when they no longer responded to gentle prodding. Animals that crawled off the plate, became desiccated on the sides of the plate, displayed extruded internal organs or died from internally-hatched progeny were

censored. Censored animals were incorporated into the data set until the day of their disqualification, as described previously (Apfeld & Kenyon *Nature* 402:804-9 (1999)). Lifespan analysis of *ced-3* mutant animals was conducted at 25°C using isogenic N2 from the Horvitz lab, kindly provided by Cori Bargmann, as a control. Lifespans of *daf-2*; *deg-1* animals were assayed at 20°C. Statview 5.0.1 (SAS) software was used to construct lifespan curves, and to determine means and percentiles. Ages given refer to days of adulthood.

5 **Lipofuscin:** Endogenous gut fluorescence was photographed using a 525 nm bandpass filter. Images were collected without automatic gain control in order to preserve the relative intensity of different animals' fluorescence. 2-, 5- and 10-day old adults were
10 photographed on the same day in order to avoid effects of light source variation on apparent fluorescence intensity.

15 **Visualization of Yolk:** The *vit-2::GFP* fusion strain (see above) was a kind gift of David Hersch and Barth Grant. Animals were allowed to age and were photographed using both Nomarski optics and epifluorescence (525 nm).

20 **Nomarski Analysis:** Animals were placed on a 2% agarose pad in M9 buffer with 2mM sodium azide and covered with a coverslip. Control experiments indicated that sodium azide did not affect the age-related phenotypes we observed. Delicate older animals of all genotypes occasionally ruptured and were lost during this process (approximately 10%). Images were captured using a CCD camera coupled to Universal Imaging Corporation's MetaMorph Imaging System (version 3.6). Image files were contrast-balanced and rotated when necessary using PhotoShop 5.0.

25 **Quantification of Tissue Damage:** *C. elegans* heads were photographed as described above. In a blind experiment, photographs of heads were given a score of 1-5, with 1 representing a youthful, unsullied appearance, and 2 through 4 denoting low, medium and high levels of overall deterioration. A rare score of 5 was assigned to animals so deteriorated as to be nearly unrecognizable.

30 Photographs of germ cells in the distal gonad also were rated on a scale of 1-5 based on these criteria. In addition, these photographs were also assigned a cumulative value that represented the presence and extent of several of the correlates of aging, e.g., graininess; large, well-separated nuclei; cavities; and a shriveled appearance. Each correlate of aging was given a separate score, which contributed to the final score. Animals could thus be compared not only by the extent of age-related degeneration, but also by the type of changes they exhibited.

Scores were assigned without knowledge of the age or genotype of the worm in the photograph. Overall scores were re-evaluated at least once, and a naive observer was asked to score a selection of photographs in a double-blind experiment.

Statistical Analysis of Tissue damage: Nonparametric analysis of head scores was conducted, using the Kruskal-Wallis Test to determine if there were significant differences between multiple groups, followed by a pairwise comparison, the Mann-Whitney test. All statistical analysis was conducted using Statview 5.0.1 (SAS) software.

C. Results

Decline of tissue integrity in aging wild-type animals

Nomarski optics are commonly used to observe the development of *C. elegans*. In young animals, the nuclei and nucleoli of all cells are readily visualized using this method. It is also possible to see the boundaries of certain cells and tissues, such as the muscles, gonad, epidermal seam cells, and certain neurons. We observed that in young adults, the cells and tissues appeared similar to those of late juvenile stages, except that the nuclear boundaries were less distinct. This lack of definition became more pronounced as the animals grew older. For example, by day 10 of adulthood, it was very difficult to see the nuclei of the epidermal cells. Neuronal nuclei, which have a wrinkled, "raisin-like" appearance, remained visible throughout the life of the animal, although they, too, grew less distinct with time. In young animals, the cytoplasm and nucleoplasm of most cells are smooth and uniform. However, as the animals grew older, both began to show signs of deterioration. Necrotic cavities of various sizes appeared, often containing vibrating particles that appeared to display Brownian motion. Tissues often acquired a curdled texture.

In older animals, the pharynx, as well as the anterior and anal regions of the intestine were frequently distended and packed with the bacteria. We wondered whether these animals were failing to chew or expel their food, or whether they might be succumbing to bacterial infection. To test this, we looked for constipation in animals grown on plates seeded with bacteria killed by UV-irradiation. We found that worms grown on killed bacteria also become highly constipated shortly before death. This suggests that very aged animals lose the ability to chew or expel their food.

Older animals also accumulated shiny, mobile patches of a substance that appeared be yolk. In young animals, yolk is transported from its site of synthesis in the intestine into the gonad, where it is incorporated into embryos. It is possible that yolk accumulates in old animals when the production of embryos ceases. We examined animals

expressing a GFP-tagged yolk protein, and confirmed that this substance was in fact yolk. We also observed shiny but less-mobile material in the bodies of worms that did not show GFP fluorescence. Finally, we also observed increased intestinal autofluorescence, which is thought to be caused by lysosomal deposits of lipofuscin. Lipofuscin is a pigment that 5 progressively accumulates in aging eukaryotic tissues as a result of the oxidative degradation and autophagocytosis of cellular components. Its ubiquitous occurrence in a variety of organisms makes it a universal marker of aging.

The cellular deterioration that we observed during aging was widespread. We chose to study the head of the worm in more detail, since it is a particularly informative and 10 compact area composed of multiple tissue types. The head contains the pharynx, a neuromuscular pump that ingests and grinds bacteria, as well as nervous tissue, muscle, and surrounding epidermis. Using Nomarski optics, we were able to evaluate the general character of this body region, but, because cellular boundaries are often indistinct, we were not able to resolve individual tissue types with certainty.

15 To quantify the changes we observed, we analyzed photographs of 84 individual wild-type worm heads and assigned each animal a numerical value that represented the extent of damage and deterioration it exhibited (see Methods). At every age, some animals exhibited more extensive tissue deterioration than others, consistent with the fact that some animals live longer than others. Nevertheless, the average level of tissue 20 deterioration in the overall population increased steadily with age in a statistically-significant manner. Together these findings indicate that the tissues of *C. elegans* deteriorate in a progressive fashion as the animals grow older. This tissue decline can account for the decreased mobility and the flaccid appearance of old worms that are visualized with a low power-dissecting microscope, and is likely to contribute to the death of the animals.

25

Tissue deterioration occurs in the germline of aging wild-type animals

The only cells that are able to divide in *C. elegans* adults are the germline stem cells. Thus it was particularly interesting to ask whether signs of age-related tissue 30 deterioration were present in this tissue. We found that the germlines of older animals showed dramatic signs of aging. The germline of *C. elegans* is a multinucleate syncytium; however, in young animals the boundaries of individual nuclei are easy to see. We found that older animals often had fewer nuclei within the gonad, that the boundaries of these nuclei were often ragged. In addition, the surrounding tissue often acquired a granular texture. Occasionally the nuclei were enlarged and appeared to be cellularized, suggesting that they

might have entered meiosis. These changes became apparent at approximately the fifth day of adulthood and increased with age. Our findings indicate that integrity of the germline declines during the life of the animal. Germline deterioration was quantified as described above, by assigning photographs of individual animals a value that reflected the condition of the tissue. Two-day, five-day and ten-day-old animals exhibited progressively more extensive degeneration, and these differences were statistically significant ($p = .0003$).

5

Mutations in the insulin/IGF-1 pathway change the rate at which both mitotic and post-mitotic tissues age

10 To ask how mutations in the insulin/IGF-1 signaling system influence tissue aging, we examined long-lived *daf-2(e1370)* and *daf-2(mu150)* mutants and short-lived *daf-16(mu86)* mutants at different ages. The *daf-2(e1370)* allele has been characterized previously (Dorman *et al.*, *Genetics* 141:1399-1406 (1995); Gems *et al.*, *Genetics* 150:129-155 (1998); Larsen *et al.*, *Genetics* 139:1567-1583 (1995)). At 25°, this mutant is
15 uncoordinated and produces progeny late in life (up to day 40; data not shown). It appears dark when viewed with a dissecting microscope. We found that these animals were shorter than normal animals (1.0 vs 1.4 mm, $p < 0.0001$), and their bodies were thinner (47 μ m vs 54 μ m, $p = 0.002$). In contrast, the *mu150* allele, which we isolated in a screen for long-lived
20 mutants, is also long lived but appears much healthier than *e1370*. Unlike *daf-2(e1370)* animals, which all become dauers at 25°C, only approximately 30% of *daf-2(mu150)* animals become dauers at this temperature. In addition, *mu150* animals moved normally and did not produce progeny late in life. Their bodies were not dark like *e1370* mutants, but resembled wild type, indicating that fat production and longevity can be uncoupled. They were only slightly shorter than normal (1.2 vs 1.4 mm, $p = 0.01$), and not thinner (53 μ m vs 54 μ m, $p = 0.45$). Overall, this mutant appears remarkably similar to wild type in terms of its behavior
25 and morphology. We also examined short-lived animals carrying the *daf-16* null mutation *mu86*. We were particularly interested to learn whether the signs of aging that we observed in wild-type animals were present in these mutants, or whether they might age in an entirely new way. In addition, since these mutations change the lifespan of the animal, we wanted to know whether they might change the rate at which the tissues aged.
30

We found that the quality of tissue deterioration in both *daf-2* and *daf-16* mutants resembled that of wild type, suggesting that both types of mutants age in a normal way but at a different rate. All of the mutants displayed increased lipofuscin-like intestinal fluorescence at relatively old ages. We found that both the somatic tissues and the germlines

of *daf-2(mu150)* and *daf-16* mutants exhibited age-related damage that appeared identical to that seen in wild-type. *daf-2(e1370)* mutants also aged in a manner similar to wild type, but they exhibited high levels of autofluorescence throughout life, and had less yolk in the body cavity than wild type, even at advanced ages (perhaps because yolk was still exported into progeny). Also, the frequency of constipation and bacterial packing in the pharynx was markedly reduced in *daf-2(e1370)* mutants, although it was present in some infirm individuals.

5 Although the character of the tissue deterioration we observed in *daf-2* mutants was similar to that of wild type, we found that the rate of this deterioration was 10 dramatically slowed. For example, in *daf-2(e1370)* mutants, the outlines of epidermal nuclei were clearly visible until at least 20 days of adulthood, compared to approximately 5 days in wild type. In addition, it was not until *daf-2* animals were approximately 20 days old that we began to see the cavities and "curdled" tissues that were so common in 5-day old wild-type animals.

15 We quantified the extent of tissue damage by scoring photographs of individual heads and germ cells in the distal gonad. Nonparametric analysis of scores assigned to *daf-2* and wild type animals of the same age confirmed our impression that *daf-2* animals exhibited far less age-related damage in both tissues. We also examined the tissues of *daf-16* mutants, whose lifespans are slightly shorter than normal at 25° C. In general, *daf-16* animals did not look dramatically older than age-matched wild type animals. On the 20 second day of adulthood, wild type and *daf-16* heads looked marginally older than heads of *daf-2* mutants ($p = 0.04$). By the fifth day of adulthood, the difference in head scores between wild type or *daf-16* and either *daf-2* allele was highly significant ($p < 0.0001$). *daf-16* and wild-type heads and germ cells were indistinguishable at early ages; it was only on the 25 tenth day of adulthood that *daf-16* mutant animals began to look significantly older than wild type when scores of heads ($p = 0.01$) and germ cells ($p = 0.02$) were compared. *daf-16* animals at this age will all be dead within two days, whereas wild type worms have a longer life expectancy.

30 The cavities we saw in old wild-type animals resembled the necrotic cell death seen in certain neurodegeneration mutants, such as the *deg-1* sodium-channel mutants. If *daf-2* mutations delay the appearance of necrotic cavities during aging, they might be expected to suppress or ameliorate these degenerations in *deg-1* animals. However, we found that *daf-2*; *deg-1* double mutants displayed both an abundance of necrotic cavities at a young age and a

long, *daf-2*-like lifespan. This suggests that *daf-2* may not directly suppress necrotic cell death, but instead may act to delay an age-dependent process that causes necrosis.

Apoptosis is unlikely to influence aging in *C. elegans*

In vertebrates, apoptosis, or programmed cell death, occurs throughout life and plays an important role in development and cellular growth control. In *C. elegans*, 131 cells undergo apoptosis, during development. It is reasonable to think that apoptosis, could potentially influence the lifespan of the organism. We looked for apoptotic cells, which have a characteristic refractile appearance (Sulston *et al.*, *Developmental Biology* 56:110-156 (1977)), in the somatic tissues of wild type and mutant animals during aging, and failed to observe them. Apoptotic cell death does occur in early meiotic cells of the germline (Gumienny *et al.*, *Development* 126:1011-1022 (1999)). To test the role of apoptosis in a more definitive way, we examined the lifespans of *ced-3* mutants, which lack a caspase that is required for apoptosis in *C. elegans*. In *ced-3* mutants, cells that should die instead remain alive and, at least in some cases, differentiate into functional cells. We reasoned that if programmed cell death influences organismal death, mutants defective in apoptosis should have abnormal, possibly extended, lifespans. However, we found that *ced-3* mutants had lifespans that were indistinguishable from those of wild type. We conclude that apoptosis does not play a significant role in the regulation of wild-type lifespan.

20

Example 2: *C. elegans* mutants that extend lifespan

Using libraries representing double stranded RNAs from Chromosome I (Fraser *et al.*, *Nature* 408:325-330 (2000)) and Chromosome II, genes that extend the lifespan of *C. elegans* when inhibited were identified. The gene encoded include mitochondrial respiratory proteins, glycolytic proteins, a GTPase, and three genes of unknown function.

A. *Screen for Lifespan Enhancing Genes.*

Animals were cultured on bacterial strains expressing double stranded RNA in a bacterial feeding library. (Kamath *et al.*, *Genome Biol.* 2:research0002.1-0002.10 (2000); Fraser *et al.*, *Nature* 408:325-330 (2000)). The library was made as follows. The sequence of the *C. elegans* genome is known and has been used to identify predicted genes. DNA fragments corresponding to predicted genes were cloned into a feeding vector L4440 between two T7 bacterial promoters in inverted orientation. The library was then transformed into a

bacterial strain carrying IPTG-inducible expression of T7 polymerase. Expression of the dsRNA was induced by addition of IPTG.

C. elegans were cultured on bacterial strains expressing dsRNA corresponding to predicted gene sequences from Chromosomes I or II. Worm lifespan was monitored. A sterile *C. elegans* strain (fem-1, fer-2 double mutant) was used to allow the lifespan of a single generation to be analyzed. To analyze lifespan, after reaching adulthood worms were transferred to new plates at least once every seven days. Worms were scored as dead if they failed to respond to being prodded three times with a wire loop. Lifespan data was entered into the Statview statistical data management program for analysis.

10

B. Inhibiting Metabolic Pathways Results in Enhanced Lifespan.

In the present invention, several metabolic genes were identified that influence the lifespan of *C. elegans* in an RNAi screen of genes located on chromosome I. All of the genes on chromosome I were expressed as double-stranded RNA in a bacterial feeding library (see, e.g., Fraser *et al.*, *Nature* 408:325-330 (2000)). *C. elegans* were cultured on each of these bacterial strains, and lifespan was monitored. Inhibition of any of four metabolic genes extended lifespan significantly.

Respiratory chain mutants

During a systematic screen of a *C. elegans* Chromosome I RNAi library it was discovered that animals grown on bacteria expressing *atp-3* double-stranded RNA lived much longer than normal (Fraser *et al.*, *Nature* 408:325 (2000)). Lifespan analysis was conducted at 25°C as described previously (Kenyon *et al.*, *Nature* 366:461 (1993)). The pre-fertile period of adulthood was used as t=0 for lifespan analysis. Strains were grown at 20°C for at least two generations before use in lifespan analysis. Statview 5.0.1 (SAS) software was used for statistical analysis and to determine means and percentiles. In all cases, *p* values were calculated using the logrank (Mantel-Cox) method.

atp-3 encodes a component of the mitochondrial ATP synthase (Complex V). In addition, we found that RNAi of three genes encoding components of the mitochondrial respiratory chain also extended lifespan. These were *nuo-2*, which encodes a component of complex I (NADH/ubiquinone oxidoreductase), *cyc-1*, which encodes a component of complex III (cytochrome C reductase) and *cco-1*, which encodes a component of complex IV (cytochrome C oxidase). Results are shown in Table 1.

Table 1
Statistical analysis of adult lifespans

<u>Strain/treatment</u>	Mean ± s.e.m. (days)	75th percentile (days)*	Number of animals that died/total†	P
N2 animals grown at 25°C on bacteria containing:				
Vector (α)	15.2 ± 0.4	18	48/52	
<i>daf-2</i> dsRNA	20.1 ± 0.9	26	55/71	<0.0001§
Complex I (<i>nuo-2</i>) dsRNA	21.6 ± 0.9	25	51/54	<0.0001§
Complex IV (<i>cco-1</i>) dsRNA	24.5 ± 0.7	27	67/69	<0.0001§
Complex V (<i>atp-3</i>) dsRNA	22.1 ± 0.9	30	54/54	<0.0001§
Vector (β)	13.6 ± 0.5	17	65/69	
Complex III (<i>cyc-1</i>) dsRNA	25.4 ± 0.9	29	65/69	<0.0001‡
<i>gpi-1</i> dsRNA	18.2 ± 0.5	22	63/67	<0.0001‡
Antimycin A Treatments				
<i>fer-15(b26); fem-1(hc17)</i> + ethanol	16.6 ± 0.6	20	57/61	
<i>fer-15(b26); fem-1(hc17)</i> + 0.1 μ M Antimycin A	20.4 ± 0.7	25	51/61	<0.0001‡
Lifespans of <i>fer-15(b26); fem-1(hc17)</i> animals grown at 25°C on bacteria containing the following dsRNA for the indicated times:				
Vector, larval and adult	16.6 ± 0.6	20	57/64	
Complex III (<i>cyc-1</i>) dsRNA, larval and adult	24.0 ± 1.0	29	50/68	<0.0001△
Complex III (<i>cyc-1</i>) dsRNA, adult only	16.9 ± 0.6	20	62/63	0.6585△
Complex V (<i>atp-3</i>) dsRNA, larval and adult	25.2 ± 0.8	29	61/80	<0.0001△
Complex V (<i>atp-3</i>) dsRNA, adult only	15.7 ± 0.5	18	55/64	0.1714△
<i>gpi-1</i> dsRNA, larval and adult	20.2 ± 0.8	25	68/71	<0.0001△
<i>gpi-1</i> dsRNA, adult only	16.3 ± 0.7	20	61/63	0.6075△
<i>daf-16(mu86)</i> animals grown at 25°C on bacteria containing:				
Vector (α)	9.8 ± 0.2	11	45/47	
<i>daf-2</i> dsRNA	9.7 ± 0.2	10	55/62	0.0792#
Complex I (<i>nuo-2</i>) dsRNA	13.7 ± 0.3	15	59/60	<0.0001#
Complex IV (<i>cco-1</i>) dsRNA	14.6 ± 0.4	18	60/60	<0.0001#
Complex V (<i>atp-3</i>) dsRNA	14.5 ± 0.6	18	35/37	<0.0001#
Vector (β)	9.2 ± 0.3	10	60/69	
Complex III (<i>cyc-1</i>) dsRNA	16.1 ± 0.3	17	43/53	<0.0001∞
<i>gpi-1</i> dsRNA	10.8 ± 0.3	12	57/64	<0.0001∞

<i>daf-2(e1370)</i> animals shifted to 25°C as L4 and grown on bacteria containing:				
Vector	25.1 ± 1.2	34	55/58	
Complex I (<i>nuo-2</i>) dsRNA	48.5 ± 1.4	54	47/60	<0.0001†
Complex III (<i>cyc-1</i>) dsRNA	41.5 ± 1.8	54	52/60	<0.0001†
Complex IV (<i>cco-1</i>) dsRNA	48.3 ± 1.4	56	50/62	<0.0001†
Complex V (<i>atp-3</i>) dsRNA	40.8 ± 3.2	54	46/59	<0.0001†
<i>gpi-1</i> dsRNA	34.5 ± 1.0	39	58/60	<0.0001†
Alteration of the reproductive system of N2 animals grown at 20°C on bacteria containing:				
intact + vector	22.3 ± 0.5	24	62/80	
intact + Complex IV (<i>cco-1</i>) dsRNA	28.9 ± 1.4	38	63/80	<0.0001‡
germline ablation + Complex IV (<i>cco-1</i>) dsRNA	44.8 ± 1.9	53	44/46	<0.0001‡
whole-gonad ablation + Complex IV (<i>cco-1</i>) dsRNA	31.7 ± 1.5	40	41/44	<0.0001‡
Alteration of the reproductive system of <i>clk-1(qm30)</i> animals grown at 20°C:				
N2	19.8 ± 0.5	24	132/159	
<i>clk-1(qm30)</i>	31.0 ± 1.2	37	67/80	<0.0001†
<i>clk-1(qm30)</i> germline ablation	37.5 ± 2.4	49	49/55	<0.0001†
<i>clk-1(qm30)</i> whole-gonad ablation	19.9 ± 0.8	23	45/50	0.4549†

* The 75th percentile is the age when the fraction of animals alive reaches 0.25.

† The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded or bagged were censored at the time of the event. This step incorporated those worms into the data set until the censor date, and was necessary to avoid the loss of information; for example, if a 50-day-old animal crawls off the plate, it is important to include that information in the data set, as that animal was long lived. Control and experimental animals were cultured in parallel and plates were changed at the same time. The logrank (Mantel-Cox) test was used to test the hypothesis that the survival functions among groups were equal. P values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time.

§ Compared with N2 worms cultured on HT115 bacteria containing an empty plasmid vector at 25°C. Vector (α), *daf-2*, Complex I, IV and V dsRNA experiments were conducted at the same time.

¶ Compared with N2 worms cultured on HT115 bacteria containing an empty plasmid vector at 25°C. Vector (β), Complex III, and *gpi-1* dsRNA experiments were conducted at the same time.

‡ Compared with control *fer-15(b26); fem-1(hc17)* double mutants grown on equivalent amount of solvent (ethanol) at 25°C.

5 Δ Compared with control *fer-15(b26); fem-1(hc17)* double mutants cultured on bacteria containing an empty plasmid vector at 25°C.

Compared with *daf-16(mu86)* mutants cultured on bacteria containing an empty plasmid vector at 25°C. Vector (α), *daf-2*, Complex I, IV and V dsRNA experiments were conducted at the same time.

10 ∞ Compared with *daf-16(mu86)* mutants cultured on bacteria containing an empty plasmid vector at 25°C. Vector (β), Complex III and *gpi-1* experiments were conducted at the same time.

15 ¶ Compared with *daf-2(e1370)* mutants cultured on bacteria containing an empty plasmid vector shifted to 25°C as L4 larvae.

¥ Compared to N2 worms with an intact reproductive system cultured on bacteria containing an empty plasmid vector at 20°C.

20 ✓ Compared to N2 worms with an intact reproductive system cultured on OP-50 bacteria at 20°C.

The protein ATP3 (Genbank # AAB37654.1) is a member of the ATP synthase delta family and is 43% identical to human ATP50. NUO2 (Genbank # AAB52474.1) protein is 56% identical to human NDUFS3. CYC1 (Genbank # CAA99820.1) is 50% identical to human CYC1. CCO1 (Genbank # CAB03002.1) is 35% identical to human COX5B. GPI-1 (Genbank # CAB60430.1) is 68% identical to human GPI.

30 We also found that treatment of wild-type animals with the drug antimycin A, which inhibits complex III (Slater, *Trends Biochem Sci.* 8:239 (1983)), increased lifespan as well.

OP-50 bacteria were spread onto NG plates and allowed to grow for two days at room temperature. 50 µl of 0.01 M Antimycin A (Sigma) dissolved in 100% ethanol was dropped onto bacterial lawn and allowed to dry overnight in a fume hood at room temperature. Eggs of *fer-15(b26); fem-1(hc17)* double mutants, which become sterile adults at 25°C, were added to the dried plates and incubated at 25°C. Animals were transferred to new Antimycin A plates every 4 days. Control worms (ethanol only) were handled in the same manner (Table 1).

Because RNAi reduces the level of wild-type mRNA, these treatments were all predicted to decrease the rate of electron transport and ATP production. We measured ATP levels as follows. For each time point, 750 *fer-15(b26); fem-1(hc17)* double mutants were grown at 25°C, to inhibit progeny production. Each time point was repeated in triplicate. The collected worms were washed four times with S-basal buffer, resuspended in cell lysis buffer and quickly frozen in liquid N₂. All samples were stored at -80°C and processed on the same day. A Roche ATP Bioluminescent HSII kit was used to measure ATP concentrations with an OPTOCOMP I luminometer. ATP concentrations were normalized to absolute protein concentrations. A BioRad protein assay kit was used to measure protein concentrations using a Perkin Elmer MBA2000 spectrophotometer.

ATP levels were reduced 60-80% in animals subjected to complex III (*cyc-1*) or ATP synthase (*atp-3*) RNAi, and 40-60% in animals treated with complex I (*nuo-2*) or complex IV (*cco-1*) RNAi. Thus, these perturbations all decrease the rate of mitochondrial respiration.

In addition to extending lifespan, respiratory-chain RNAi affected growth and behavior. First, the animals were smaller than normal, suggesting that mitochondrial respiration promotes growth. Because the small animals were well proportioned, it seems likely that a metabolite whose level is regulated by mitochondrial respiration acts as a signal to control body size. Small body size itself is unlikely to cause lifespan extension, because mutants defective in *daf-4*, which encodes a Smad protein (Estevez *et al.*, *Nature* 365:644 (1993)), are small but not long lived (Tissenbaum *et al.*, *Nature* 410:227 (2001)). Respiratory-chain RNAi also decreased the rate of growth to adulthood, as well as the rates of pharyngeal pumping (eating) and defecation (Table 2). In addition, the animals moved much more slowly than normal (Table 2). Mutations in *mev-1*, *gas-1*, *clk-1* and *isp-1* all reduce the rates of development and movement (Ishii *et al.*, *Nature* 394:694 (1998); Hartman *et al.*, *Mech Aging Dev.* 122:1187 (2001); Wong *et al.*, *Genetics* 139:1247 (1995); Feng *et al.*, *Developmental Cell* 1:663 (2001)) but do not affect body size, suggesting that the level of respiratory activity in our RNAi-treated animals may be lower than in these mutants.

Table 2
Timing of biological processes in RNAi-treated and *clk-1* mutant animals

dsRNA or Other Treatment	Feeding Rate (pumps/min. ± s.d.)*	Defecation Rate (seconds/defecation ± s.d.)†	Mobility (swim strokes/min. ± s.d.)‡	Length of post-embryonic development (hours ± s.d.)‡
Vector	201 ± 9	41 ± 3	130 ± 6	43 ± 2
Complex I(<i>nuo-2</i>)	159 ± 8	80 ± 4	90 ± 2	69 ± 3
Complex III(<i>cyc-1</i>)	161 ± 10	81 ± 1	75 ± 13	88 ± 2
Complex IV(<i>cco-1</i>)	144 ± 9	79 ± 2	94 ± 3	68 ± 2
Complex V(<i>atp-3</i>)	124 ± 17	87 ± 5	90 ± 9	96 ± 2
<i>gpi-1</i>	198 ± 8	43 ± 3	127 ± 6	43 ± 2
<i>clk-1(qm30) intact</i>	161 ± 36	69 ± 6	77 ± 9	
<i>clk1(qm30) germline-ablated</i>	140 ± 20	50 ± 8	91 ± 7	
<i>clk-1(qm30) gonad-ablated</i>	117 ± 22	63 ± 9	93 ± 5	

5 * Mean ± s.d. The number of pharyngeal pumps observed in a one-day-old adult animal in 1 minute at 20°C (n ≥ 8).

10 † Mean ± s.d. The time required to complete one defecation cycle. A minimum of three consecutive defecations was recorded for each trial at 20°C (n ≥ 5).

Mean ± s.d. The number of swim cycles completed in 1 minute of a one-day-old adult animal at 20°C (n ≥ 5).

15 ‡ Mean ± s.d. Time (hours) to adulthood at 20°C (n ≥ 25).

Because RNAi can be administered at different times during the life of an animal, we were able to ask whether mitochondrial respiration acts in an ongoing fashion throughout life to influence aging. To do this, we transferred animals onto bacteria expressing respiratory-chain dsRNA at different ages (Dillin *et al.*, *Nature*, submitted).

20 Surprisingly, we found that if we allowed the animals to grow to adulthood on normal bacteria, and then shifted them as young adults onto bacteria expressing respiratory-chain dsRNA, their lifespans were not extended (Table 1). To determine whether RNAi lowered respiratory-chain activity in these animals, we assayed their ATP levels and found that they were reduced to the same extent as in animals exposed to respiratory-chain dsRNA from 25 hatching. Thus respiratory-chain RNAi does decrease mitochondrial activity in adult

animals. Together these findings indicate that the respiratory chain acts during development and not during adulthood to influence lifespan.

We also asked whether initiating RNAi during adulthood decreased behavioral rates. The rate of movement we observed was the same as that of control animals, but the 5 rate of pumping was decreased (Table 3). Interestingly, however, whereas animals treated with RNAi from the time of hatching pumped in a slow but steady fashion, animals treated as young adults exhibited bursts of rapid pumping followed by pauses. Possibly these pauses were caused by transient depletion of ATP.

10

Table 3
Pumping rates of larva and adults treated with respiratory-chain RNAi

RNAi Treatment	Feeding Rate (pumps/min ± s.d.)*			
	Day 2	Day 3	Day 4	Day 5
Vector	118 ± 15 (0/8)	140 ± 21 (0/8)	145 ± 24 (0/8)	131 ± 49 (1/10)
Complex III (<i>cyc-1</i>) larval + adult	97 ± 21 (0/10)	83 ± 12 (0/10)	84 ± 12 (0/10)	75 ± 12 (0/10)
Complex III (<i>cyc-1</i>) adult only	79 ± 62 (5/12)	112 ± 80 (3/12)	80 ± 59 (4/12)	69 ± 45 (4/12)
Complex V (<i>atp-3</i>) larval and adult	98 ± 15 (0/10)	87 ± 10 (0/10)	80 ± 9 (0/10)	75 ± 8 (0/10)
Complex V (<i>atp-3</i>) adult only	88 ± 80 (6/14)	98 ± 64 (5/14)	83 ± 59 (4/14)	89 ± 54 (4/14)

* Mean ± s.d. The number of pharyngeal pumps observed in an adult animal in 1 minute at 25°C. Number in parentheses is the number of animals that produced less than 50 pumps per minute/ total number of animals tested.

15

Interaction of respiratory chain mutants with the DAF 16 pathway.

The insulin/IGF-1 signaling pathway affects lifespan (Guarente *et al.*, *Nature* 408:25 (2000)). Reducing the activity of DAF-2, an insulin/IGF-1 receptor homolog, or 20 downstream signaling components, extends lifespan approximately two-fold. This lifespan extension requires activity of the forkhead-family transcription factor DAF-16.

However, we found that *daf-16(mu86)* null mutants lived much longer than normal when subjected to respiratory-chain RNAi (Table 1). In addition, we found that the already long lifespans of *daf-2(e1370)* mutants were nearly doubled again when the animals 25 were cultured on any of the four bacterial strains expressing respiratory chain dsRNA (Table 1). Moreover, unlike reduction of respiratory chain activity, reduction of insulin/IGF-1

signaling is known to cause a significant increase in ATP levels (Braeckman *et al.*, *Curr Biol*, 9:493 (1999)).

Finally, both *daf-2* and *daf-16* act exclusively in adults to regulate lifespan (Dillin *et al.*, *Nature*). Together these findings indicate that respiratory-chain RNAi does not 5 increase lifespan by inhibiting the DAF-2 pathway.

Interaction of respiratory chain mutants with the reproductive pathway.

In *C. elegans*, removing the germline extends lifespan (Hsin *et al.*, *Nature* 399:362 (1999)). This lifespan extension is not caused by sterility *per se*, because animals in 10 which the somatic gonad as well as the germline are removed have normal lifespans (Hsin *et al.*, *Nature* 399:362 (1999); Kenyon *et al.*, *Nature* 366:461 (1993)).

We found that animals treated with respiratory-chain RNAi were either sterile or had small broods. The longevity of these animals was not likely to be caused by reduced 15 germline activity, however, because the lifespan extension caused by germline ablation is *daf-16* dependent (Hsin *et al.*, *Nature* 399:362 (1999)).

To test whether the reproductive system could influence lifespan in these RNAi-treated animals, we removed either their germlines or the entire reproductive systems. The germlines or the entire reproductive systems of complex IV (*cco-1*) RNAi-treated 20 animals were removed by killing Z2 and Z3 or Z1 and Z4, respectively. Laser ablations were performed on newly hatched animals using a nitrogen-pumped dye laser, which produces a wavelength of 440 nm. Successful ablation was confirmed by examining the reproductive systems of adult animals with a dissecting microscope. Worms were anaesthetized with 1.0 - 1.5 M NaN₃ during ablation, a treatment that had no effect on lifespan. The germline and 25 whole-gonad of ablation *clk-1(qm30)* mutants was performed as described above, in two experiments, with similar results.

In addition to the laser ablation experiments described above, the germlines of respiratory-chain RNAi-treated animals were ablated genetically using *glp-1(e2141ts)* mutant worms, which, at 25°C, produce only a few germ cells and live longer than wild type. Mean 30 lifespans of *glp-1(e2141ts)* mutant worms grown at 25°C on bacteria expressing the following dsRNA were: Control (empty vector), 15.0 ± 0.7; Complex I (*nuo-2*) dsRNA, 19.2 ± 0.7 (*p* < 0.0001); Complex III (*cyc-1*) dsRNA, 19.7 ± 1.0 (*p* < 0.0001); Complex IV (*cco-1*) dsRNA, 22.9 ± 0.6 (*p* < 0.0001); and Complex V (*aip-3*) dsRNA, 23.0 ± 0.6 (*p* < 0.0001)). As with wild type, germline ablation further extended lifespan, whereas whole-gonad ablation

did not (Table 1). Thus the reproductive system of these animals appeared to influence lifespan in a normal way.

We also examined the role of the reproductive system in *clk-1* mutants. As with animals subjected to respiratory-chain RNAi, ablating the germlines of *clk-1* mutants caused an increase in lifespan. However, removing the whole gonad of *clk-1(qm30)* mutants produced an unexpected result; it completely abolished their lifespan extensions (Table 1). Thus the longevity of these animals is entirely dependent on the presence of the reproductive system. Interestingly, ablation of the gonad did not rescue the slow growth or behavioral phenotypes of *clk-1(qm30)* mutants (Table 2). This finding is significant, because it indicates that the longevity of these mutants is not likely to be caused by their slow rates of growth or behavior.

Glycolytic Pathway Mutants

In our Chromosome I RNAi screen, we also found that lowering the activity of a gene that functions in glycolysis, glucose phosphate isomerase (*gpi-1*), increased lifespan approximately 20% (Table 1). GPI-1 (Genbank # CAB60430.1) is 68% identical to human GPI. The *C. elegans* genome contains only one glucose phosphate isomerase, implying that these animals have lowered rates of glycolysis. Consistent with this, we found that these animals had reduced levels of ATP.

In vertebrates, PGI is required not only for glycolysis but also for the survival of sensory neurons (Chaput *et al.*, *Nature* 332:454 (1988); Faik *et al.*, *Nature* 332:455 (1988)). Sensory neurons are known to influence the lifespan of *C. elegans* (Apfeld *et al.*, *Nature* 402:804 (1999)). However, when we used a fluorescent dye (DiI) to visualize many sensory neurons, we failed to detect any abnormalities (data not shown). In addition, whereas the lifespan extension of sensory mutants is largely *daf-16* dependent (Apfeld *et al.*, *Nature* 402:804 (1999)), this was not the case for animals treated with *gpi-1* RNAi (Table 1). Together these findings suggest that *gpi-1* RNAi does not increase lifespan by affecting sensory neurons.

Like respiratory chain RNAi, *gpi-1* RNAi further extended the lifespans of *daf-2(e1370)* mutants (Table 1). Interestingly, unlike animals treated with respiratory-chain RNAi, these animals appeared healthy and were normal in size. They had wild-type rates of growth to adulthood and rates of behavior (Table 2). In addition, animals treated with *gpi-1* RNAi as adults were not long-lived (Table 1), suggesting that, like respiratory-chain activity, *gpi-1* may function during development to affect lifespan.

C. *Inhibiting a GTPase Results in Enhanced Lifespan.*

Using the screen described above, a Rab-like GTPase was identified from a Chromosome I library. The name of the gene is T23H2.5 and its accession number is AAC482001. Analysis of the mutant demonstrated that inhibition of the gene extended lifespan by 25%. The inhibition of lifespan was independent of the Daf2/16 pathway.

D. *Inhibiting *llw* genes results in an enhanced lifespan.*

An RNAi screen of a chromosome II library identified three novel genes that extend lifespan when inhibited. *llw-1*, gene Y54G11A.8, contains a TPR-like domain and its accession number is CAA22452 (SEQ ID NO: 7). *llw-3*, gene Y48E1B.1, contains a proline-rich domain and its accession number is CAB07688 (SEQ ID:9). Inhibition of *llw-2*, gene F59E12.10, also resulted in enhanced lifespan, and its accession number is AAB54251 (SEQ ID NO:8). Table 1 shows the result of dsRNA expression corresponding to these genes.

15

Table 4
Inhibition of *llw* genes enhances *C. elegans* lifespan

Gene	dsRNA expressed	% worms dead at day 20
	Vector only	88%
<i>llw-1</i>	Y54G11A.8	
<i>llw-2</i>	F59E12.10	37%
<i>llw-3</i>	Y48E1B.1	52%
<i>llw-4</i>	F45H10.4	35%

Example 3: *C. elegans* mutants that shorten lifespan

20 Using the Chromosome I library described previously, *C. elegans* were fed bacteria expressing dsRNA and screened for shortened lifespan. A cursory examination using objective aging criteria allowed immediate discarding of 6 out of 7 short-lived RNAi-treated strains from further consideration as possible progeric animals. Moreover, the one remaining clone had a dramatic phenocopy of normal aging.

25 The progeric RNAi candidate encoded a homologue of HSF (heat-shock factor), a transcription factor that activates expression of heat-shock genes in response to heat shock and oxidative stress. The HSF-1 gene is Y53C10.12 and the accession number is

CAA22146. As heat shock factor genes are highly conserved, this family of genes therefore is shown to be involved in aging, including human homologs of HSF family genes, e.g., HSF-1.

In a second experiment, over-expression of HSF-1 gene in *C. elegans* caused an increased adult lifespan. The HSF-1 gene has been cloned and constructed into a plasmid that can be used to inject into the worm. The HSF-1 gene was ectopically over-expressed in the worms that carry this plasmid. Compared to wild type worms, these worms have an increased lifespan.

10 Example 4: Screening for genes that affect aging using microarrays

In *C. elegans*, aging is regulated in one instance by an insulin/IGF-1 pathway. An insulin receptor homolog, DAF-2 activates a PI 3 kinase pathway which in turn appears to inhibit the function of DAF-16, a forkhead-family transcription factor. *daf-2* mutants are long lived, whereas *daf-16* and *daf-16; daf-2* mutants are short lived. Previous experiments have shown that this *daf-2* pathway functions non-cell autonomously, meaning that it must regulate the expression of one or more genes that either encode or regulate the production of a downstream signal or hormone. In addition, there must be a cellular pathway that responds to this second hormone, and finally genes that encode proteins directly involved in the aging process.

20 Microarrays were constructed using *C. elegans* DNA primers purchased from Research Genetics. The arrays were then used to analyze gene expression profiles in *daf-2* and *daf-16* mutants. The expression of a number of genes varied in long-lived (*daf-2*) or short-lived (*daf-16* and *daf-16; daf-2*) *C. elegans* mutants (see Table 5). The activity of genes identified using microarray analysis was then inhibited using RNAi (Table 6). The genes thus identified include hormones that activate the *daf-2* pathway, several encoding cytochrome P450 proteins, e.g., genes involved in lipid and steroid biosynthesis, the melatonin synthesis gene (human homolog ASMTL, Accession No. NM_004192), insulin and insulin-like peptides (e.g., ins-7), heat shock factors, catalases, stress-response genes, neuronal genes, oxidative stress protection genes, antibacterial and antifungal genes, vitellogenins, and metabolic genes (see Figures 5-12).

Example 5: Screening for genes and compounds that affect aging using a DAF-16/GFP fusion protein.

A DAF16-GFP fusion was constructed as described in Lin *et al.*, *Nature Gen.* 28:139-145(2001). DAF16 is a transcription factor and in normal animals is localized throughout cells. Using the DAF16-GFP fusion protein, localization of DAF-16 in long lived animals was studied. In animals with extended lifespans, the DAF-16/GFP fusion was localized to the nuclei.

The following extended lifespan mutants were analyzed: *daf-2* mutants and germline ablated animals. DAF-2 is an insulin /IGF-1 receptor homologue. In *daf-2* mutants, 10 lifespan is extended in a *daf-16* dependent manner. When expressed in *daf-2* mutants, the DAF-16/GFP fusion was localized in most nuclei throughout larval development and in adults. In germline ablated animals, the DAF-16/GFP fusion was localized in the nuclei of intestinal cells and to a lesser extent the nuclei of other cell types also.

Localization of DAF-16/GFP is used to screen for drugs or mutations that extend lifespan. Localization of DAF-16/GFP is also used to determine whether a mutation extends lifespan by perturbing the insulin/IGF-1 pathway, germline pathway, or other aging regulatory pathway.

Example 6: Screening for genes and compounds that affect aging using an SOD-3/GFP fusion protein

The *C. elegans* SOD-3 (superoxide dismutase) gene is regulated by the insulin/IGF-1 system. Mutants in the insulin/IGF1 pathway overexpress SOD-3. When an SOD-3/GFP was expressed in long-lived mutants of the insulin/IGF1 pathway, the mutants had a higher level of GFP fluorescence, especially in the intestine, than did wild-type worms expressing the same fusion protein.

Worms expressing the SOD-3/GFP fusion protein are screened for extended lifespan by assaying for intense fluorescence patterns. Screens are done for mutants in the insulin/IGF-1 pathway, for long-lived germline signaling mutants, and for respiratory chain mutants. Screens are also done to identify drugs that affect the aging process.

30

TABLE 5

CF512 WL Sept	description	mean	std err	% vector	% avg V
C04F6.1	vit-5	15.92	0.626	121.5267	103.7134
C17G1.4	muc-1	11.38	0.477	86.87023	74.13681

CF512 WL Sept	description	mean	std err	% vector	% avg V
C17G10.5	Ent.His. N-acetylmuraminidase	13.53	0.846	103.2824	88.14332
C18B2.3		12.22	0.495	93.28244	79.60912
C40H5.1		12.68	0.736	96.79389	82.60586
C44E4.2		13.39	0.79	102.2137	87.23127
C54G4.6	ASMTL	16.92	0.758	129.1603	110.228
C55B7.4	AcylCoADH	13.5	0.885	103.0534	87.94788
Y54G11A.6	ctl-1	16.3	0.748	124.4275	106.1889
Y54G11A.5b	ctl-2	13.53	0.808	103.2824	88.14332
Daf-16		13	0.519	99.23664	84.69055
Daf-2		17.1	0.834	130.5344	111.4007
F08B1.1	vhp-1 phosphatase	15.5	0.644	118.3206	100.9772
F21F3.3	farnesyl cystein carboxyl methyltransferase	12.73	0.501	97.17557	82.9316
F32A5.5	aquaporin	14.37	0.63	109.6947	93.61564
F35H12.2	PTB/PID GAP	14	0.562	106.8702	91.20521
F48D6.4		14.07	1.06	107.4046	91.66124
F56G4.3	F-box domain sim.to pes-2	14.84	0.684	113.2824	96.67752
F59D8.2	vit-4	12.5	0.541	95.41985	81.43322
H16D19.1	C-type lectin	13.22	0.778	100.916	86.12378
H22K11.1	asp-3	15	0.708	114.5038	97.71987
K07A1.7		15.12	0.617	115.4198	98.50163
K10B3.8	gpd-2	14.05	0.679	107.2519	91.53094
K11D2.2	ASAH acid ceramidase	14.86	1.024	113.4351	96.80782
R03E9.1	mdl-1 tf	15.8	0.522	120.6107	102.9316
T01A4.1	Npr1-like	13.19	0.734	100.687	85.92834
T07D10.4	C-type lectin	12.96	1.03	98.9313	84.42997
T10B9.1	Cyt P450	10.9	0.443	83.20611	71.00977
T25C12.2	like T08A9.9 antibacterial	13.72	0.874	104.7328	89.38111
T25C12.3	EGF/lectin	14.34	1.02	109.4656	93.4202
vector		13.1	0.532	100	85.34202
ZK1320.2		14.63	0.459	111.6794	95.30945
ZK270.2	FERM domain/band 4.1	10.54	0.536	80.45802	68.6645
ZK355.E		15.2	0.585	116.0305	99.0228

CF512adult	description	mean	std err	% vector	% avg V
C04F6.1	vit-5	17.15	0.587	103.1269	111.7263844
C17G1.4	muc-1	18.67	0.411	112.267	121.6286645
C17G10.5	Ent.His. N-acetylmuraminidase	15.724	0.471	94.55201	102.4364821
C18B2.3		15.83	0.544	95.18942	103.1270358
C40H5.1		18.2	0.542	109.4408	118.5667752
C44E4.2		19.33	0.536	116.2357	125.9283388
C54G4.6	ASMTL	18.41	0.803	110.7035	119.9348534
C55B7.4	AcylCoADH	17.27	0.676	103.8485	112.5081433
Y54G11A.6	ctl-1	16.71	0.567	100.4811	108.8599349
Y54G11A.5b	ctl-2	16.38	0.341	98.49669	106.7100977
Daf-16		14.53	0.49	87.37222	94.65798046
Daf-2		21.36	0.953	128.4426	139.1530945

CF512adult	description	mean	std err	% vector	% avg V
F08B1.1	vhp-1 phosphatase	13.43	0.506	80.75767	87.49185668
F21F3.3	farnesyl cystein carboxyl methyltransferase	15.86	0.449	95.36981	103.3224756
F32A5.5	aquaporin	15.43	0.468	92.78413	100.5211726
F35H12.2	PTB/PID GAP	18.87	0.972	113.4696	122.9315961
F48D6.4		17	0.567	102.2249	110.7491857
F56G4.3	F-box domain sim.to pes-2	18.58	0.791	111.7258	121.0423453
F59D8.2	vit-4	17.22	0.657	103.5478	112.1824104
H16D19.1	C-type lectin	15.34	0.541	92.24293	99.93485342
H22K11.1	asp-3	17.36	0.498	104.3897	113.0944625
K07A1.7		15.68	0.559	94.28743	102.1498371
K10B3.8	gpd-2	17.36	0.629	104.3897	113.0944625
K11D2.2	ASAHH acid ceramidase	15.77	0.607	94.82862	102.7361564
R03E9.1	mdl-1 tf	16.96	0.536	101.9844	110.4885993
T01A4.1	Npr1-like	17.25	0.48	103.7282	112.3778502
T07D10.4	C-type lectin	14.93	0.496	89.77751	97.26384365
T10B9.1	Cyt P450	15.83	0.448	95.18942	103.1270358
T25C12.3	like T08A9.9 antibacterial	17.8	0.83	107.0355	115.9609121
vector	EGF/lectin	16.63	0.468	100	108.3387622
ZK1320.2		11.72	0.435	70.47505	76.35179153
ZK270.2		18.3	0.771	110.0421	119.218241
ZK355.E	FERM domain/band 4.1	15.52	0.462	93.32532	101.1074919

CF512 Dec.	description	mean	std err	% vector	% avg. V
AC3.7	UDP-glucuronosyl transferase	14.78	0.365	101.2329	96.28664
B0213.15	Cyt P450	14.35	0.365	98.28767	93.48534
B0554.1		12.67	0.417	86.78082	82.54072
B0554.6	like AK6.11	12.43	0.4	85.13699	80.9772
C04F6.1	vit-5	12.52	0.372	85.75342	81.56352
C17G1.4	muc-1	12.21	0.41	83.63014	79.54397
C17H12.8		15.16	0.325	103.8356	98.76221
C24B9.4	DUF32 domain	14.12	0.376	96.71233	91.98697
C32F10.9		13.59	0.381	93.08219	88.5342
C32H11.10	DUF141	12.92	0.493	88.49315	84.16938
C32H11.12	DUF141	12.1	0.38	82.87671	78.82736
C46F4.2	long-chain fatty acid-Coenzyme A ligase 4	13.33	0.386	91.30137	86.84039
C54D10.1	GST	13.56	0.371	92.87671	88.33876
C54G4.6	ASMTL/Maf-like	12.81	0.422	87.73973	83.45277
C55B7.4	Acyl-CoA DH	13.04	0.431	89.31507	84.95114
Daf-16		8.34	0.288	57.12329	54.33225
Daf-2		16.017	0.235	109.7055	104.3453
F10D2.9	fat-7	14.71	0.33	100.7534	95.83062
F11A5.12	short-chain DH-reductase;hydroxysteroid 17-beta DH	13.93	0.407	95.41096	90.74919
F28D1.3	thaumatin	12.28	0.442	84.10959	80
F28D1.5	thaumatin	12.29	0.463	84.17808	80.06515

F38E11.2	hsp-12.6		14.79	0.383	101.3014	96.35179
F49A5.6	thaumatin		14.43	0.341	98.83562	94.00651
F55G11.5			12.1	0.356	82.87671	78.82736
K04E7.2	pep-2		10.6	0.315	72.60274	69.05537
K06A11.1			15.16	0.374	103.8356	98.76221
K07C6.4	Cyt P450		12.13	0.411	83.08219	79.0228
K10D11.1			14.29	0.337	97.87671	93.09446
K11G9.6	mtl-1		13.16	0.429	90.13699	85.7329
K12G11.3	Alcohol DH		12.58	0.396	86.16438	81.9544
T10B9.1	Cyt P450		10.77	0.309	73.76712	70.16287
T13F2.1	fat-4		13.12	0.369	89.86301	85.47231
T16G12.1	aminopeptidase		14.25	0.367	97.60274	92.83388
T20G5.7			15.7	0.314	107.5342	102.2801
T22G5.2	lbp-7		14.116	0.349	96.68493	91.96091
Vector			14.6	0.372	100	95.11401
W06D12.3	fat-5		13.69	0.429	93.76712	89.18567
W08D2.4	fat-3		12.13	0.241	83.08219	79.0228
Y38H6C.5	Zn knuckle domain		13.25	0.37	90.75342	86.31922
Y49E10.8			15.85	0.291	108.5616	103.2573
ZK1251.2	ins-7		16.4	0.19	112.3288	106.8404
ZK384.4			12.6	0.401	86.30137	82.08469

CF596	description	mean	std err	% vector	CF596	% vector
B0213.15	Cyt P450	26	0.5	111.9242	B0213.15	111.9242
C02A12.4	N-acetylmuramidase	18.63	0.855	80.19802	vector	100
C05E4.4		18.933	0.913	81.50237	K12G11.3	91.08911
C24B9.4	DUF23	18.59	0.816	80.02583	F10D2.9	82.5226
Daf-16		11.47	0.423	49.37581	C05E4.4	81.50237
Daf-2		13.88	0.659	59.75032	C02A12.4	80.19802
F10D2.9	fat-7	19.17	0.885	82.5226	T20G5.7	80.19802
F28D1.3	thaumatin	18.4	0.927	79.20792	C24B9.4	80.02583
F38E11.2	hsp-12.6	18.42	0.78	79.29402	K11G9.6	79.85364
K11G9.6	mtl-1	18.55	1.02	79.85364	F38E11.2	79.29402
K12G11.3	Alcohol DH	21.16	1.05	91.08911	F28D1.3	79.20792
T10B9.1	Cyt P450	15.36	0.813	66.12139	T10B9.1	66.12139
T20G5.7		18.63	0.938	80.19802	Daf-2	59.75032
vector		23.23	1.02	100	Daf-16	49.37581

rrf-3 20C	description	mean	std err	% vector	RNAi	% vector
C04F6.1	vit-5	17.47	0.763	116.4667	daf-2	207.3333
C32H11.12	DUF141	19.7	0.676	131.3333	ZK1251.2	155.2
C42D8.2	vit-2	18.15	0.576		121 C54G4.6	132.38
C54G4.6	ASMTL	19.857	0.658	132.38	C32H11.12	131.3333
daf-16		14.84	0.476	98.93333	ZK896.1	125.4667
daf-2		31.1	0.749	207.3333	C42D8.2	121
vector		15.02	0.613	100.1333	C04F6.1	116.4667
ZK1251.2	ins-7	23.28	0.512	155.2	vector	100.1333
ZK896.8	gcy-18	18.82	0.731	125.4667	daf-16	98.93333

CF512 25	description	mean	std err	% vector	% avg. V
C04F6.1	vit-5	14.7	0.586	86.47059	95.76547
C32H11.12		16.5	0.635	97.05882	107.4919
C42D8.2	vit-2	15.42	0.58	90.70588	100.456
C54G4.6	ASMTL	18.71	0.637	110.0588	121.8893
daf-16		11.2	0.279	65.88235	72.96417
daf-2		18.13	0.76	106.6471	118.1107
vector		17.06	0.574	100.3529	111.1401
ZK1251.2	ins-7	17.05	0.606	100.2941	111.0749
ZK896.8	gcy-18	16.41	0.695	96.52941	106.9055

vector	16.63	0.468
vector	17.06	0.574
Vector	14.6	0.372
vector	13.1	0.532
	15.3475	

TABLE 6

Upregulated in daf-2 mutants and with daf-2 RNAi, downregulated with daf-16 RNAi

Gene	Gene product/homology
C15H9.1	C15H9.1: Protein of unknown function
C53B7.3	C53B7.3: Protein with weak similarity to EGF-like repeats, has moderate similarity to <i>C. elegans</i> F46C8.4
C34C6.7	C34C6.7: Protein of unknown function
E04F6.9	E04F6.9: Protein of unknown function, has moderate similarity to <i>C. elegans</i> E04F6.8
C50F7.5	C50F7.5: Protein of unknown function, has moderate similarity to <i>H. sapiens</i> MUC1 gene product, a transmembrane mucin
Y15E3B.f	Y15E3B.f Y15E3B.f
F09F7.7	F09F7.7: Protein of unknown function
T23G7.3	T23G7.3: Protein of unknown function, has weak similarity to <i>S. cerevisiae</i> Ygr280p
F08B12.4	F08B12.4: Protein of unknown function
F47H4.10	F47H4.10: Member of an uncharacterized protein family
H14N18.1	H14N18.1: unc-23: Highly similar to mammalian BAG-2, BCL2-associated athanogene 2, a chaperone regulator
C40H1.5	C40H1.5: Member of an uncharacterized protein family
T23B3.2	T23B3.2: Protein of unknown function, has weak similarity to <i>C. elegans</i> F47B7.1
C25E10.8	C25E10.8: Member of an uncharacterized protein family
Y105C5A.12	Y105C5A.12: Protein of unknown function
B0507.8	B0507.8: Protein of unknown function, has similarity to <i>C. elegans</i> B0507.7
C25E10.9	C25E10.9: Member of an uncharacterized protein family
F38E11.1	F38E11.1: Member of the small heat shock protein family
Y51A2D.11	Y51A2D.11: Member of an uncharacterized protein family
Y51A2B.1	Y51A2B.1: Protein of unknown function, has moderate similarity to <i>C. elegans</i>

	elegans C07G3.2
C08E8.4	C08E8.4: Protein of unknown function, has weak similarity to <i>C. elegans</i> C07G3.2
C44H9.5	C44H9.5: Protein of unknown function
K10E9.1	K10E9.1: Protein of unknown function
C08F11.3	C08F11.3: Protein of unknown function, has moderate similarity to <i>C. elegans</i> F56G4.1
C01H6.6	C01H6.6: Protein of unknown function, has weak similarity to <i>S. cerevisiae</i> Yol060p
C24A11.8a	C24A11.2 C24A11.2
W06D12.3	W06D12.3: fat-5: Likely a palmitoyl-CoA delta-9 fatty acid desaturase, specific for medium chain (14)
T02B5.1	T02B5.1: Member of the carboxylesterase protein family
C05E4.9	C05E4.9: Putative ortholog of <i>S. cerevisiae</i> MLS1 gene product (Malate synthase 1, functions in glyoxylate cycle, has near identity to Dal7p)
ZC395.5	ZC395.5: Protein of unknown function
H24O09.c	H24O09.c H24O09.c
C24B9.9	C24B9.9: Protein of unknown function, has moderate similarity to <i>C. elegans</i> T04C12.1
T20G5.7	T20G5.7: Protein of unknown function, has moderate similarity to <i>C. elegans</i> T20G5.8
K07C6.4	K07C6.4: Member of the P450 heme-thiolate protein family
B0213.15	B0213.15: Member of the P450 heme-thiolate protein family
F09F7.6	F09F7.6: Protein of unknown function
R03E9.1	R03E9.1: mtl-1: Member of the MAD family of putative transcription factors, interacts with <i>C. elegans</i> MAX-1
B0238.1	B0238.1: Member of the carboxylesterase protein family
E01G4.3	E01G4.3: Protein of unknown function
M02D8.4	M02D8.4: Member of the asparagine synthetase protein family
Y6E2A.3	Y6E2A.3: Protein of unknown function, has weak similarity to <i>C. elegans</i> Y6E2A.5
F38E11.2	F38E11.2: hsp-12.6: Member of the small heat shock protein family
F17B5.1	F17B5.1: Protein with strong similarity to <i>C. elegans</i> T20D4.7 gene product [Member of the thioredoxin protein family]
K07A1.7	K07A1.7: Protein with similarity to <i>D. melanogaster</i> HDC (headcase) protein, a branching inhibitor produced by specialized tracheal cells
F48D6.4	F48D6.4: Protein of unknown function
F11A5.12	F11A5.12: Protein with similarity to estradiol 17-beta-dehydrogenases; strongly related to <i>C. elegans</i> C06B3.4, C06B3.5, F25G6.5, and C56G2.6
C06B3.4	C06B3.4: Possible estradiol 17 beta-dehydrogenase, member of a protein family
H10D18.2	H10D18.2: Putative paralog of <i>C. elegans</i> H10D18.4
Y40B10A.6	Y40B10A.e Y40B10A.e
ZK355.3	ZK355.3: Protein of unknown function
K11G9.6	K11G9.6: mtl-1: Metallothionein-related, cadmium-binding intestinal protein
C02A12.4	C02A12.4: Member of an uncharacterized protein family with weak similarity to <i>Entemeba histolytica</i> N-acetylmuraminidase
AC3.7	AC3.7: Member of the UDP-glucuronosyltransferase protein family
C54D10.3	C54D10.3: Member of an uncharacterized protein family

Upregulated in daf-2 mutants

<u>Gene</u>	<u>Gene product/homology</u>
ZK973.7	ZK973.7: Protein of unknown function, putative paralog of <i>C. elegans</i>
ZK973.7	ZK973_14.I
ZK973.7	ZK973.7: Protein of unknown function, putative paralog of <i>C. elegans</i>
ZK973.7	ZK973_14.I
W10G6.3	W10G6.3: ifa-2: Putative intermediate filament protein
ZK270.2a	ZK270.2 ZK270.2
F16H6.7	F16H6.7: Member of an uncharacterized protein family
F57H12.7	F57H12.7: Protein of unknown function, has weak similarity to <i>C. elegans</i>
	W06B11.1
F32A5.5	F32A5.5: Protein with strong similarity to human AQP9, an aquaporin; putative paralog of <i>C. elegans</i> C01G6.1 and Y69E1A.G
C17G1.4	C17G1.4: Protein with moderate similarity to <i>H. sapiens</i> MUC1 gene product, a transmembrane mucin
K12G11.4	K12G11.4: Member of the alcohol dehydrogenase protein family
K12G11.3	K12G11.3: Member of the alcohol dehydrogenase protein family
F21F3.3	F21F3.3: Protein with strong similarity to <i>S. cerevisiae</i> Ste14p, a farnesyl cysteine
T07D10.4	T07D10.4: Member of the C-type lectin protein family
H16D19.1	H16D19.1: Member of the C-type lectin protein family
K11D2.2	K11D2.2: Putative acid ceramidase, has strong similarity to human ASAII, acid ceramidase (N-acylsphingosine amidohydrolase)
C52E4.1	C52E4.1: gcp-1: Cysteine protease expressed in the intestine
W08D2.4	W08D2.4: fat-3: Protein with delta6-fatty acid-desaturase activity
F53F4.13	F53F4.13: Protein of unknown function, has weak similarity to <i>C. elegans</i>
	F20A1.10
C26C6.3	C26C6.3: Member of the zinc metalloprotease protein family, has strong similarity to <i>C. elegans</i> TOH-2, has similarity to <i>D. melanogaster</i> and human TGF-beta-like growth factors
K08F4.7	K08F4.7: gst-4: Member of the glutathione S-transferase protein family, has similarity to human and <i>D. melanogaster</i> glutathione S-transferases
VC5.3	VC5.3: Ladder protein
VC5.3	VC5.3: Ladder protein
E01A2.8	E01A2.8: Putative paralog of <i>C. elegans</i> E01A2.7
K10B3.8	K10B3.8: gpd-2: Glyceraldehyde-3-phosphate dehydrogenase
R09B5.6	R09B5.6: Member of the hydroxyacyl-CoA dehydrogenase protein family
C56A3.2	C56A3.2: Member of an uncharacterized protein family
H22K11.1	H22K11.1: asp-3: Probable aspartyl protease and an ortholog of human cathepsin D
C06G8.1	C06G8.1: Protein of unknown function, has strong similarity to <i>C. elegans</i>
	K02D7.5, has similarity to <i>D. melanogaster</i> SLV (saliva), a putative transmembrane protein

Downregulated in daf-2 mutants and with daf-2 RNAi, upregulated with daf-16 RNAi

<u>Gene</u>	<u>Gene product/homology</u>
M60.1	M60.1: Putative serine proteinase, has strong similarity to human placental

serine proteinase, P11
 ZK6.10: Protein of unknown function, putative paralog of *C. elegans*
 ZK6.11
 C49C3.9: Protein of unknown function
 F55G11.7: Member of an uncharacterized protein family
 F55G11.8: Member of an uncharacterized protein family
 K10D11.1: Member of an uncharacterized protein family
 C32H11.4: Member of an uncharacterized protein family
 Y46C8_103.a
 F56D6.2: Member of the phospholipase A2 receptor protein family
 B0365.6: Member of the C-type lectin protein family
 ZK1127.10: Member of the cystathionine gamma-lyase protein family
 C04F12.3: Protein with similarity to human B-cell CLL/lymphoma 3 (BCL3)
 T05A12.3: Protein of unknown function, has weak similarity to *C. elegans*
 R07G3.3
 C17H12.8: Member of an uncharacterized protein family
 C17B7.1: G protein-coupled receptor, member of a large subfamily that contains ODR-10 odorant response protein, no homolog found in human or *D. melanogaster*
 F15E11.1
 F15E11.12
 F22A3.6
 F58F6.2
 B0478.1: jnk-1: Neuronally expressed serine/threonine protein kinase of the MAP kinase subfamily
 F59D8.2: vit-4: Member of the vitellogenin protein family; expressed only in *C. elegans* intestinal cells
 F59D8.f F59D8.f
 ZK757.1: Protein of unknown function with similarity to a DHHC zinc finger domain, putative paralog of *C. elegans* B0546.5 protein
 R11G1.3: Member of the glutathione S-transferase protein family, has similarity to *H. sapiens* and *D. melanogaster* glutathione S-transferases
 C04F6.1: vit-5: 170 kDa yolk protein
 F41A4.1: Protein with weak similarity to *C. elegans* let-653 (Putative mucin-like protein required for development beyond the late L1 larval stage or early L2 larval stage)
 C54G4.6: Protein with similarity to human ASMTL protein, acetylserotonin N-methyltransferase-like protein
 F15E11.9 F15E11.9
 Y19D10A.j Y19D10A.j
 F56A4.j F56A4.j
 K08D8.5: Member of an uncharacterized protein family
 C32H11.1: Member of an uncharacterized protein family
 T03E6.7: Member of the thiol protease protein family
 R09H10.5: Member of the EGF-repeat protein family
 T25C12.3: Member of the EGF-repeat protein family, member of the C-type lectin family
 K12H4.7: Member of the carboxypeptidase protein family

F28H7.3	F28H7.3: Member of the lipase protein family
Y38H6C.5	Y38H6C.5: Putative Zinc finger, CCHC class protein
F57F4.3	F57F4.3: Protein of unknown function, putative paralog of <i>C. elegans</i>
	F57F4.4
F57F4.4	F57F4.4: Protein of unknown function, putative paralog of <i>C. elegans</i>
	F57F4.3
C12C8.2	C12C8.2: Member of the cystathione gamma-lyase protein family
W01A11.4	W01A11.4: Member of the galactoside-binding lectin protein family
C08F11.8	C08F11.8: Member of the UDP-glucuronosyltransferase protein family
Y38H6C.1	Y38H6C.1: Protein of unknown function, has weak similarity to <i>C. elegans</i>
M02H5.B	
K06A4.5	K06A4.5: Putative 3-hydroxyanthranilate 3,4-dioxygenase, has strong similarity to human Hs.108441 gene product (3-hydroxyanthranilic acid dioxygenase)
F23H11.7	F23H11.7: Protein of unknown function
F52G3.4	F52G3.4: Protein with weak similarity to <i>C. elegans</i> F33H12.6
F46E10.1	F46E10.1 F46E10.1
C39E9.1	C39E9.1: Member of the testis-specific protein TPX-1 like protein family
C25F6.3	C25F6.3: Member of the 4Fe-4S ferredoxins and related iron-sulfur cluster binding protein family
F52E1.5	F52E1.5: Protein of unknown function
F28B4.3	F28B4.3: Member of the EGF-repeat protein family
Y106G6H.10	Y106G6H.10: Protein of unknown function, putative paralog of <i>C. elegans</i>
Y106G6H.9	Y106G6H.9: Protein of unknown function, putative paralog of <i>C. elegans</i>
Y106G6H.10	Y106G6H.10
K06A4.1	K06A4.1: Putative zinc metalloprotease with similarity over the N-terminus to human and <i>D. melanogaster</i> TOLLOID-like proteins, has strong similarity over the middle region to <i>C. elegans</i> F56A4.K and Y19D10.A.K
B0281.5	B0281.5: Putative potassium voltage-gated channel, Shaw-related subfamily
F13A7.9	F13A7.9: Protein with moderate similarity to <i>S. cerevisiae</i> Skp1p, a component of the kinetochore complex and a component of SCF (Skp1p-cullin-F-box) complexes that target proteins for ubiquitin-dependent degradation; also has strong similarity to several other <i>C. elegans</i> proteins
Y51H7BR.2	Y51H7BR.2: Protein of unknown function, putative paralog of <i>C. elegans</i>
	Y51H7BR.1
Y38E10A.14	Y38E10A.n Y38E10A.n
Y46H3C_14.c	Y46H3C_14.c Y46H3C_14.c
Y9D1A.1	Y9D1A.1: Protein with moderate similarity to <i>C. elegans</i> Y9D1A.A
Y49E10.8	Y49E10.8: Protein of unknown function
F56G4.3	F56G4.3: Protein of unknown function, is identical to <i>C. elegans</i> PES-2
F56G4.2	F56G4.2: pes-2: Protein of unknown function, is identical to <i>C. elegans</i>
	F56G4.3
C52D10.9	C52D10.9: Protein with similarity to the SKP1 family of proteins, nearly identical to <i>C. elegans</i> C52D10.7
C52D10.7	C52D10.7: Protein with similarity to the SKP1 family of proteins, nearly identical to <i>C. elegans</i> C52D10.9
Y56A3A.15	Y56A3A.15: Member of an uncharacterized protein family

C31A11.5	C31A11.5: G protein-coupled receptor, member of unnamed subfamily with distant homology to SRG subfamily, no homolog found in human or <i>D. melanogaster</i>
F49E11.7	F49E11.7: Member of the protein phosphatase protein family
C32H11.10	C32H11.10: Member of an uncharacterized protein family
C32H11.9	C32H11.9: Member of an uncharacterized protein family
ZK6.11	ZK6.11: Protein of unknown function, putative paralog of <i>C. elegans</i>
ZK6.10	
B0554.6	B0554.6: Member of an uncharacterized protein family
F55G11.5	F55G11.5: Member of an uncharacterized protein family
C32H11.12	C32H11.12: Member of an uncharacterized protein family
F35E12.5	F35E12.5: Member of an uncharacterized protein family
C08F8.5	C08F8.5: Protein of unknown function, has weak similarity to <i>C. elegans</i>
F38H4.2	
Y62H9A.3	Y62H9A.3: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.5	
Y62H9A.4	Y62H9A.4: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.6	Y62H9A.6: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.4	
Y62H9A.5	Y62H9A.5: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.3	
F49E12.2	F49E12.2: Member of the calpain protease protein family
ZK896.8	ZK896.8: gcy-18: Protein with a cytoplasmic receptor tyrosine kinase domain and a guanylate cyclase domain, has strong similarity to human natriuretic peptide receptor NPR1 and photoreceptor-specific membrane retina guanylyl cyclase RetGC-2
T24B8.5	T24B8.5: Protein of unknown function, has weak similarity to <i>C. elegans</i>
F49F1.7	
K02H11.2	K02H11.2: G protein-coupled receptor, member of a large subfamily that contains ODR-10 odorant response protein, no homolog found in human or <i>D. melanogaster</i>
Y22F5A.5	Y22F5A.5: Member of an uncharacterized protein family with weak similarity to <i>Entereba histolytica</i> N-acetylmuramidase
C25B8.3	C25B8.3: cpr-6: Member of the Cathepsin B-like Cysteine Protease family
Y55B1AR.1	Y55B1AR.1: Protein with weak similarity to <i>C. elegans</i> W09H1.6 (Galactoside-binding lectin)
F49C12.7	F49C12.7: Member of an uncharacterized protein family
ZK896.5	ZK896.5: Member of an uncharacterized protein family
W02D9.7	W02D9.7: Protein of unknown function
F49F1.1	F49F1.1: Member of an uncharacterized protein family

SEQUENCE LISTING

SEQ ID NO:1 Cytochrome C1 C54G4.8, Accession No. CAA99820.1

5 MQRAVVQGSK RGLAALAGVT AASGMGLVYA LENSVSASGD NVHPYALPWA
 HSGPFSSFDI ASVRRGYEVY KQVCAACHSM KFLHYRHFVD TIMTEEEAKA
 EAADALINDV DDKGASIQRP GMLTDKLNP YPNKKAAAAA NNGAAPPDLS
 LMALARHGGD DYVFSLLTGY LEAPAGVKVD DGKAYNPYFP GGIISMPQL
 FDEGIEYKDG TPATMSQQAK DVSAFMHWAA EPFHDTTRKKW ALKIAALIPF
 10 VAVVLIYGKR HIWSFTKSQK FLFKTVKGRE PPKAQ

SEQ ID NO:2 NADH oxidoreductase T10E9.7, Accession No. AAB52474.1

MDSEILNYPI HRFLDSSLG QFIGPEGSNI YKIEKYNKVA LDIWKNDEEN
 15 SNVRITGPYW NLKSALNDVL ELVSTIRNKN QRYKFEMPSK DIGFLIGKNG
 AKINEIKLSS NVDVHFERNN ENRDNGETDG RVKMLGSVIR QAVSRQIVRN
 SPISTTAAVA QTNQTGDKKE SPKKPTIWKI DEHKRERLAN FGKYAAECLP
 KFVQKVQFAA GDELELLIHP SGVVPVLSFL KGNHSAQFTN LTFITGMDVP
 TRKNRLEVIY SLYSVRFNAR VRVRTYTDEI APIDSATPVF KGADWFEREV
 20 YDMYGVWFNN HPDLRRILTD YGFEGHPFRK DYPLSGYNEV RYDPELKRVV
 YEPSELAQEF RKFDLNTPWE TFPAFRNQSI TSGYETILEV AEPTPATPQN K

SEQ ID NO:3 ATP synthase F27C1.7, Accession No. AAB37654.1

25 MAQLMKRGFS TSAALAKAQL VKTPIQVHGV EGRYAAALYS AGHKQNKLDO
 ISTDLNNSVRS VYKDNKKFQE FVLDPTLKAN KKTAIEAIS TKLGLTKETG
 NFLGLLAENG RLNKLESVVS SFESIMRAHR GELFVQVTSV EELSSSNQKA
 LSDALKIGK SGQKLTVTYA VKPSILGGLV VTIGDKYVDL SIASRVKKYK DALATAI

30 SEQ ID NO:4 cytochrome C oxidase F26E4.9, Accession No. CAB03002.1

MAQLAKTAVA ALSKKLVAPA AVARRTLATE ASPEDYGYYP DPLEHATGRE
 KKMILLARLAG DDRYEPKVYY RAEASTKQKP NLVPSHYDFR IIGCMCEQDS
 GHVNFMFTIRK GDPKRCECGH WFKGVDADPE SI

SEQ ID NO:5 phosphoglucose isomerase Y87G2A.8, Accession No.

CAB60430.1

5 MSLSQDATFV ELKRHVEANE KDAQLLELFE KDPARFEKFT RLFATPDGDF
 LFDFSKNRIT DESFQLLMRL AKSRGVEESR NAMFSAEKin FTENRAVLHV
 ALRNRA NRPI LVDGKDVM PD VNRVLAHMKE FCNEIIISGSW TGYTGKKITD
 VVNIGIGGSD LGPLMVTESL KNYQIGPNVH FVSNVDGTHV AEVTKKLNAE
 TTLFIIASKT FTTQETITNA ETAKEWFLAK AGDAGAVAKH FVALSTNVTK
 10 AVEFGIDEKN MFEFWDWVG G RYSLWSAIGL SIAVHIGFDN YEKL LDGAFS
 VDEHFVNTP L EKNIPVILAM IGVLYNNIYG AETHALLPYD QYMHRFAAYF
 QQGDME SNGK FVTRHGQRVD YSTGPIVWGE PGTNGQHAFY QLIHQGTRLI
 PADFIAPVKT LNPIRGGLHH QILLANFLAQ TEALMKGKTA AVAEAEKLSS
 GMSPESIAKI LPHKVFEGNK PTT SIVLPVV TPFTLGALIA FYEHKIFVQG
 15 IIWDICSYDQ WGVELGKQLA KVIQPELASA DTVTSHDAST NGLIAFIKNN A

SEQ ID NO:6 GTPase gene T23H2.5, Accession No. AAC48200.1

MARRPYDMLF KLLLIGDSGV GKTCILYRFS DD AFNTTFIS TIGIDFKIKT
 20 IELKGKKIKL QIWDTAGQER FHTITTSYYR GAMGIMLVYD ITNAKSFDNI
 AKWLRNIDEH ASE DVVKMIL GNKCDMSDRR VVS RERGEKI AQDHGIFSHE
 TSAKLN VHVD TAFYDLAEAI LAKMPDSTDE QSRDTVNPVQ PQRQSSGGC C

SEQ ID NO:7 product of LLW-1 gene Y54G11A.8, Accession No.

25 CAA22452

MLPSMSKLCT STVRPVAAAF STGTTRQHHS SGRSRQQHHR HGGSGGKTNG
 GGRWSRYGKS AATGGTTVLA LSWMTTIKDV LGIEKVQLDA DPLKEKVQKS
 WLYRKRRQYD DAIQVLQLAL EEAERKEDM PITRVYDEMA NTFYEKMNL
 30 EADKYFRIVI QRLVQLHGKK DFDPEFIGVS LKLADILAH R GDLESAESGF
 KHCVRQRQMKV MEEHMKKFSV AHGALVEDRH TVDTFGPMYT DPIALFGMTL
 EAYANFLINY CGETRMAEVE EYIDEVMKIS YQIYGASSAH TINMLNNFGA
 TLVLKNRFEL AKKYLAIGVD RILYVNECAH MLPGYYCNYA ESLFHTGQKN
 EA LEFARKAV QMSRSGDDRV RHYTQNFLND LEKDINRGKP KSWWFF

SEQ ID NO:8 product of LLW-2 gene F59E12.10, Accession No. AAB54251

MNASSRTKPA IDLNKVPPID HHRTAVTFNC LIMKMTEMLN NFGNKMEDIL
 5 EKAEQSLDTA DRKLRLMESK LAGMSLEDKS TTATPSSAPE IDEIHESNPS
 SSQIVEETVE EKPEEHTTV LIKDDPAYSK YFKMLKLGVL EAGVIQKMK
 EGVDPSILKR GDEPSRPQAQ TSRNYESSGE STASFSDSD

SEQ ID NO:9 LLW-3 gene Y48E1B.1, Accession No. CAB07688

10 MYHVPLIPRD AGREETIFRI NQSLQKLLRV SDEIFDRVEH RITRIHGKAE
 AIDRRTEVLE KKLESIQESD KVITFTLPRQ LPKLPEEPPT STSLFRINID
 TEHFPGSEEL PAFRRADDHV LRPCEPIDFT YELNKPDKFF LTSQVLKEYE
 QKGWERYKKR LLGGLRELSR SPEHIAELFY AGTSIPAFEG VSGDFSKKAL
 15 DADDDGGTSR SGRTTDELAQ LRLHEQLLED TALSSTLMQE DSLDDNHPLA
 FRINFNEKKK KTAKMVEMPD SLPNLKGHAH DFTLRDPEID EDRLLDILPA
 DDQIPEASEP TEAEADAPTT FILPPPPPPM KLDPSQPAA TPVEITEIPP
 IISPPAPPBP PPPPPPPPPP QTPSASSSVT FSPTKSVDGG RSDLMAAIRA
 AGGAGNAKLS RIAEKPKRG KFDGILESSA LLGASETPRN SAPAPDGCGG
 20 GGDLMSALSK ALDARRKAIN GKVEAQPPAK VSSTIPAPPN FDDEEW
 D

SEQ ID NO:10 product of LLW-4 gene F45H10.4 , Accession No. CAB04386

25 MRIIACSLIASLIPTVIGLKSNRSCFHYSCLEATEANLKQCAGGTAISLTLEAKDVNR
 DLVKYRALEFVGCQDRLLKEVVFESLQILVNEDARECFEKLPESSKRVEEFTDSCDYVQPV
 SRNASKGDALQCLIEFKQDREYCESLLECCPDHTRCGERNAVSLSYQNARVKAEQIVYSMI
 SCIVVNDPRFLREGARLQSLRDPYRNAGVPFLRPDAYTEARITRRLALTSTATLSQRERFL
 KKYSQIRQVVAQNLFGQQQLTRPVEDLVTETSSKLAVEAPEETTTQEETTTDASEVTTK
 AVEEATEEVTEATEATEAPVATTKESSEMHVNTIRNMIRSASEKDLSKYVTLISEGKFSEL
 30 FELAEQKKLTLSKFDLKSSKMAKLKDLINEALSEKEKSGEIEQAMEKFKEKPEKSELVAME
 DKDTPAVFTISDSLKHKKAEAKLAHTIVSRNVVEAENAIEKEVVEPKAEEKVKEEDVKAVA
 EEKKEEKPGKLPMKIEKLEKPVDTKSENHELKKVLDCKERALLVESEIKNTAEETKPKVES
 FKSEETTVAILDDMPALEKEESAEEKETTGEPTTEAAVETTEASETPKPEAKPELLSNLEDV
 LTLLTTPETETIEGSGEREETTSAPAAEATSEITLLKSSSDVAVIENVKRIRPRTEQTHCQQ
 35 YASCWQTVLDYEQQCDRKYSTEVLSHGIDDSEIILNILHNSSISHHEIVLKACLRPLDRSVHS
 TLKQLVIQRGVRKACLELGRNKIAVTDSEEALCNETIPSTAIADEFISSEHVRSQSNHLTC
 RAKLEPIRETCISIVRNCCASVDTCDNYISSSPVKKLETEAIRRLVKKQNDSETKMLQTLSYI
 HEQLSNPSRRRRFYYH

SEQ ID NO:11 product of HSF-1 gene Y53C10A.12, Accession No.

CAA22146

MQPTGNQIQQ NQQQQQQQLIM RVPKQEVS VS GAARRYVQQA PPNRPPRQN H
5 QNGAIGGKKS SVTIQEVPNN AYLETLNKSG NNKVDDDKLP VFLIKLWNIV
EDPNLQSIVH WDDSGASFHI SDPYLFGRNV LPHFFKHNNM NSMVRQLNMY
GFRKMTPLSQ GGLTRTESDQ DHLEFSHPCF VQGRPELLSQ IKRKQSARTV
EDKQVNEQTQ QNLLEVVAEM RAMREKAKNM EDKMNKLTK E NRDMWTQMGS
MRQQHARQQQ YFKKLLHFLV SVMQPGLSKR VAKRGVLEID FCAANGTAGP
10 NSKRARMNSE EGPyKDVC DL LESLQRETQE PFSRRFTNNE GPLISEVTDE
FGNSPVGRGS AQDLFGDTFG AQSSRYSDGG ATSSREQSPH PIISQPQSNS
AGAHGANEQK PDDMYMGSGP LTHENIHRGI SALKRDYQGA SPASGGPSTS
SSAPSGAGAG ARMAQKRAAP YKNATRQMAQ PQQDYSGGFV NNYSGFMPSD
PSMIPIYQPSH QYLQPHQKLM AIEDQHHPTT STSSTNADPH QONLYSPTLGL
15 SPSFDRQLSQ ELQEYFTGTD TSLESFRDLV SNHNWDDFGN NVPLDDDEEG
SEDPLRQLAL ENAPETSNYD GAEDLLLFDNE QQYPENGFDV PDPNYLPLAD
EEIFPHSPAL RTPSPSDPNL V

WHAT IS CLAIMED IS:

- 1 1. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:
 - 3 (i) contacting the compound with a polypeptide, wherein the polypeptide is
4 encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5 encoding a polypeptide comprising an amino acid sequence selected from the group
6 consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2),
7 ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose
8 isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2
9 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), LLW-4 (SEQ ID NO:10) and HSF-1 (SEQ ID
10 NO:11), and human homologues thereof; and
11 (ii) determining the functional effect of the compound upon the
12 polypeptide.
- 1 2. The method of claim 1, wherein the polypeptide is encoded by a
2 nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a
3 polypeptide comprising an amino acid sequence selected from the group consisting of:
4 cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase
5 (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ
6 ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8),
7 LLW-3 (SEQ ID NO:9), and LLW-4, (SEQ ID NO:10) or human homologues thereof.
- 1 3. The method of claim 1, wherein the polypeptide is encoded by a
2 nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a
3 polypeptide comprising an amino acid sequence of HSF-1 (SEQ ID NO:11), or human
4 homologues thereof.
- 1 4. The method of claim 1, wherein the functional effect is determined
2 *in vitro*.
- 1 5. The method of claim 4, wherein the functional effect is determined
2 by measuring enzymatic activity.
- 1 6. The method of claim 4, wherein the functional effect is determined
2 by measuring ligand, substrate, or cofactor binding to the polypeptide.

1 7. The method of claim 4, wherein the functional effect is determined
2 by measuring binding to a promoter sequence by the polypeptide.

1 8. The method of claim 1, wherein the polypeptide is expressed in a
2 mitochondrial extract or an isolated mitochondrial membrane.

1 9. The method of claim 1, wherein the polypeptide is expressed in a
2 eukaryotic host or host cell and the polypeptide is contacted with the compound in a
3 living cell.

1 10. The method of claim 9, wherein the host cell is derived from *C.*
2 *elegans*, mouse, rat, or human.

1 11. The method of claim 9, wherein the host is *C. elegans*, mouse, rat,
2 or human.

1 12. The method of claim 9, wherein the functional effect is a
2 determined by measuring ligand, substrate, or cofactor binding to the polypeptide.

1 13. The method of claim 9, wherein the functional effect is determined
2 by measuring transcriptional activation.

1 14. The method of claim 9, wherein the functional effect is determined
2 by evaluating age-associated parameters.

1 15. The method of claim 9, wherein the functional effect is determined
2 by evaluating expression of an age-associated gene.

1 16. The method of claim 14, wherein the age-associated parameter is
2 lifespan.

1 17. The method of claim 1, wherein the modulation is inhibition of
2 aging.

1 18. The method of claim 17, wherein inhibition of aging occurs by
2 activation of an HSF polypeptide encoded by a nucleic acid that hybridizes under
3 stringent conditions to a nucleic acid encoding a polypeptide comprising an amino acid
4 sequence of HSF-1 (SEQ ID NO:11), or human homologues thereof.

1 19. The method of claim 17, wherein inhibition of aging occurs by
2 inhibition of a polypeptide encoded by a nucleic acid that hybridizes under stringent
3 conditions to a nucleic acid encoding a polypeptide comprising an amino acid sequence
4 selected from the group consisting of: cytochrome C₁ (SEQ ID NO:1), NADH
5 oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase
6 (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6),
7 LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), and LLW-4
8 (SEQ ID NO:10) or human homologues thereof

1 20. The method of claim 1 wherein the polypeptide is recombinant.

1 21. The method of claim 1, wherein the compound is an antibody, an
2 antisense molecule, or a small molecule.

1 22. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide, wherein the polypeptide is
4 encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5 encoding a polypeptide comprising an amino acid sequence selected from the group
6 consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2),
7 ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose
8 isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2
9 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), LLW-4 (SEQ ID NO:10) and HSF-1 (SEQ ID
10 NO:11), and human homologues thereof;

11 (ii) determining the functional effect of the compound upon the
12 polypeptide; and

13 (iii) contacting a host or host cell expressing the protein and evaluating an
14 age-associated parameter in a host or host cell, thereby identifying a compound that
15 modulates aging.

1 23. The method of claim 22, wherein the polypeptide is recombinant.

1 24. The method of claim 22, wherein the functional effect is a physical
2 effect.

1 25. The method of claim 22, wherein the functional effect is a chemical
2 effect.

1 26. The method of claim 22, wherein the functional effect is a
2 phenotypic effect.

1 27. The method of claim 22, wherein the functional effect is
2 determined *in vitro*.

1 28. The method of claim 22, wherein the functional effect is
2 determined in a eukaryotic host organism or host cell.

1 29. The method of claim 22, wherein the age-associated parameter is
2 lifespan.

1 30. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide that is a component of the
4 mitochondrial respiratory chain; and
5 (ii) determining the functional effect of the compound upon the
6 polypeptide.

1 31. The method of claim 30, wherein the polypeptide is expressed in a
2 mitochondrial extract or an isolated mitochondrial membrane.

1 32. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide, wherein the polypeptide is
4 encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5 listed in Table 5 or Table 6, or a nucleic acid encoding a polypeptide listed in Table 5 or
6 Table 6, or human homologues thereof; and
7 (ii) determining the functional effect of the compound upon the
8 polypeptide.

1 33. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide, wherein the polypeptide is
4 encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5 listed in Table 5 or Table 6, or a nucleic acid encoding a polypeptide listed in Table 5 or
6 Table 6, or human homologues thereof

(ii) determining the functional effect of the compound upon the polypeptide; and

1 34. A compound that modulates an aging process, wherein the
2 compound is identified by the method of claim 1.

1 35. The compound of claim 34, wherein the compound is an antibody,
2 an antisense molecule, or a small molecule.

1 36. The compound of claim 34, wherein the compound is antimycin or
2 an analog thereof.

1 37. A method of increasing lifespan or treating premature aging in a
2 subject, the method comprising the step of administering to the subject an effective
3 amount of a compound identified using the method of claim 1.

38. The method of claim 37, wherein the compound is antimycin.

1 39. A method of increasing lifespan or treating premature aging in a
2 subject, the method comprising the step of administering to the subject an effective
3 amount of a compound that modulates the expression of a polypeptide comprising an
4 amino acid sequence selected from the group consisting of: cytochrome C₁ (SEQ ID
5 NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3),
6 cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5),
7 GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ
8 ID NO:9), LLW-4 (SEQ ID NO:10) and HSF-1 (SEQ ID NO:11), and human
9 homologues thereof.

1 40. The method of claim 37 or 39, wherein the aging process is
2 abnormal.

1 41. The method of claim 40, wherein the abnormal aging process is
2 selected from Werner syndrome, Hutchinson-Guilford disease, Bloom's syndrome,
3 Cockayne's syndrome, ataxia telangiectasia, and Down's syndrome.

1 42. The method of claim 37 or 39, wherein the aging process is normal.

1 43. The method of claim 37 or 39, further comprising the step of
2 evaluating an age-associated parameter of the subject.

1 44. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:

3 (i) providing a library of nucleic acids, each nucleic acid of the library comprising
4 a segment of a gene of an organism; and

5 (ii) for each member of the library,

6 (a) generating double-stranded RNA (dsRNA) from the respective member
7 of the library,

8 (b) providing the dsRNA to a cell or one or more cells of the organism to
9 provide a ds-RNA treated cell or ds-RNA treated organism, and

10 (b) monitoring an age-associated parameter of the ds-RNA treated cell or
11 the ds-RNA treated organism.

1 45. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:

3 (i) evaluating a relationship between presence or abundance for each species of a
4 plurality of RNA or protein species with respect to age of a cell or organism;

5 (ii) producing or delivering a double-stranded RNA to a cell or one or more cells
6 of an organism, the RNA corresponding to a species whose presence or abundance is
7 correlated with age; and

8 (iii) monitoring an age-associated parameter of the cell or the one or more cells or
9 the organism.

1 46. A method of identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting a test compound to a living or biochemical system that comprising a
4 *C. elegans* target protein selected from the group consisting of: a respiratory chain
5 component, a heat shock promoter activator, LLW-1, LLW-2, LLW-3, LLW-4, a small
6 GTPase, a protein in Table 5, and a protein in Table 6; and
7 (ii) evaluating a property associated with the target protein; and
8 (iii) evaluating an aging-associated parameter of a *C. elegans* organism contacted
9 with the test compound.

1 47. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:

3 (i) providing a nematode in which activity of a target protein is reduced in the
4 organism by RNA interference;
5 (i) expressing a gene encoding a candidate mammalian protein that is
6 heterologous to the organism; and
7 (iii) evaluating an age associated parameter of the organism.

1 48. A *C. elegans* nematode that (1) expresses a heterologous gene in at
2 least some cells, the heterologous gene encoding a respiratory chain component or
3 glycolysis component that is non-identical to the corresponding endogenous respiratory
4 chain component or glycolysis component, or a functional domain thereof; and (2) is
5 deficient in at least some cells for an endogenous activity provided by the corresponding
6 endogenous respiratory chain component or glycolysis component.

1 49. A method comprising: assessing an age-associated parameter of the
2 nematode of claim 48.

1 50. A *C. elegans* nematode that (1) has a deficiency in at least some
2 cells for an endogenous activity, the deficiency generated by dsRNA in the cells, and (2)
3 has an average lifespan of at least 40% greater than an otherwise identical nematode
4 without the deficiency.

1 51. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:

3 (i) providing the nematode of claim 50;
4 (ii) introducing a heterologous gene that encodes a heterologous polypeptide into
5 the nematode;
6 (iii) expressing the heterologous gene in the nematode or a progeny of the
7 nematode under conditions wherein the heterologous polypeptide is produced; and
8 (iv) monitoring an age-associated parameter of the nematode or the progeny of the
9 nematode.

1 52. The method of claim 51, further comprising contacting a test
2 compound to the nematode or the progeny prior to or during the monitoring.

RNAi of respiratory chain and glycolysis genes increases life span

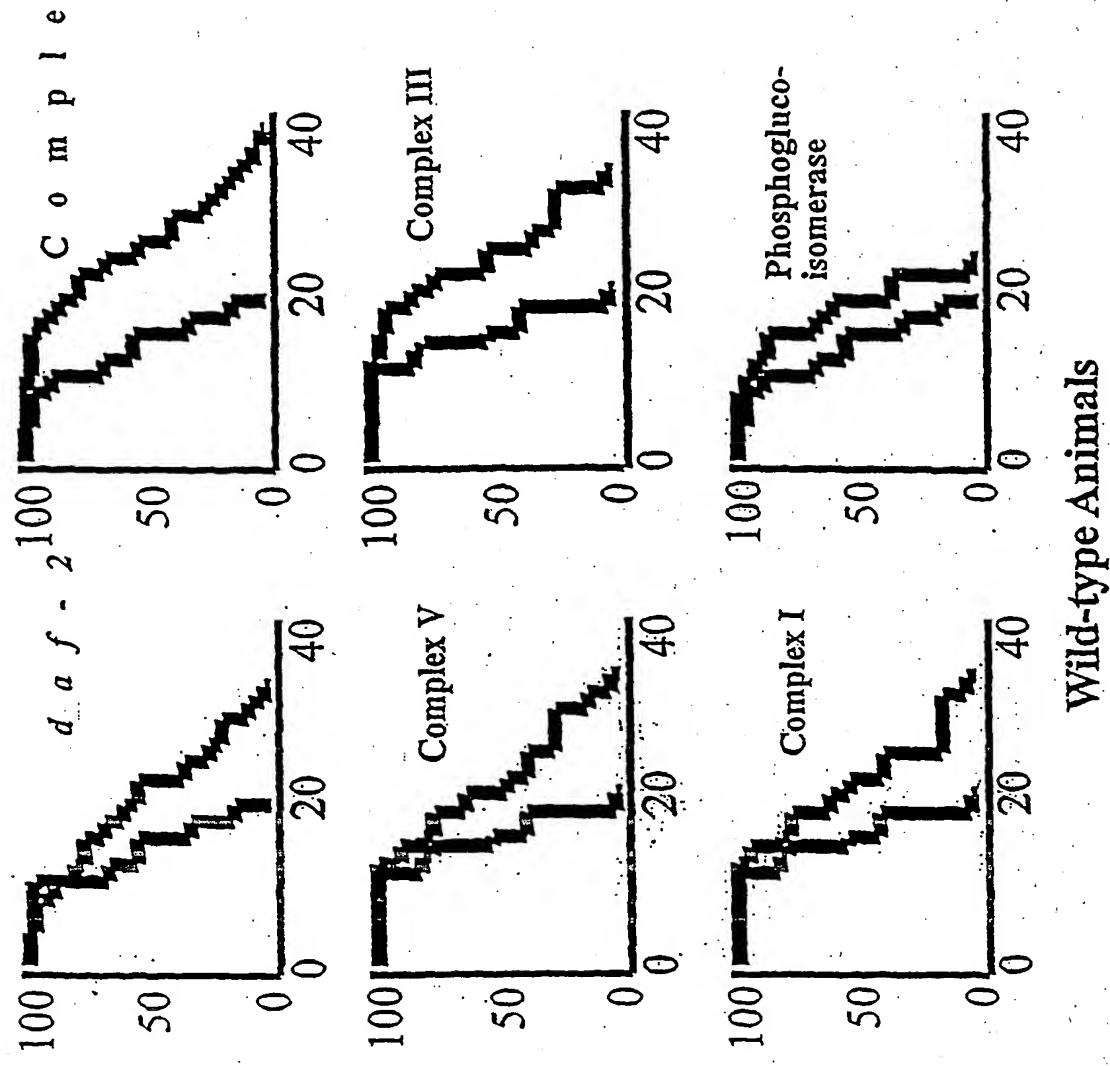
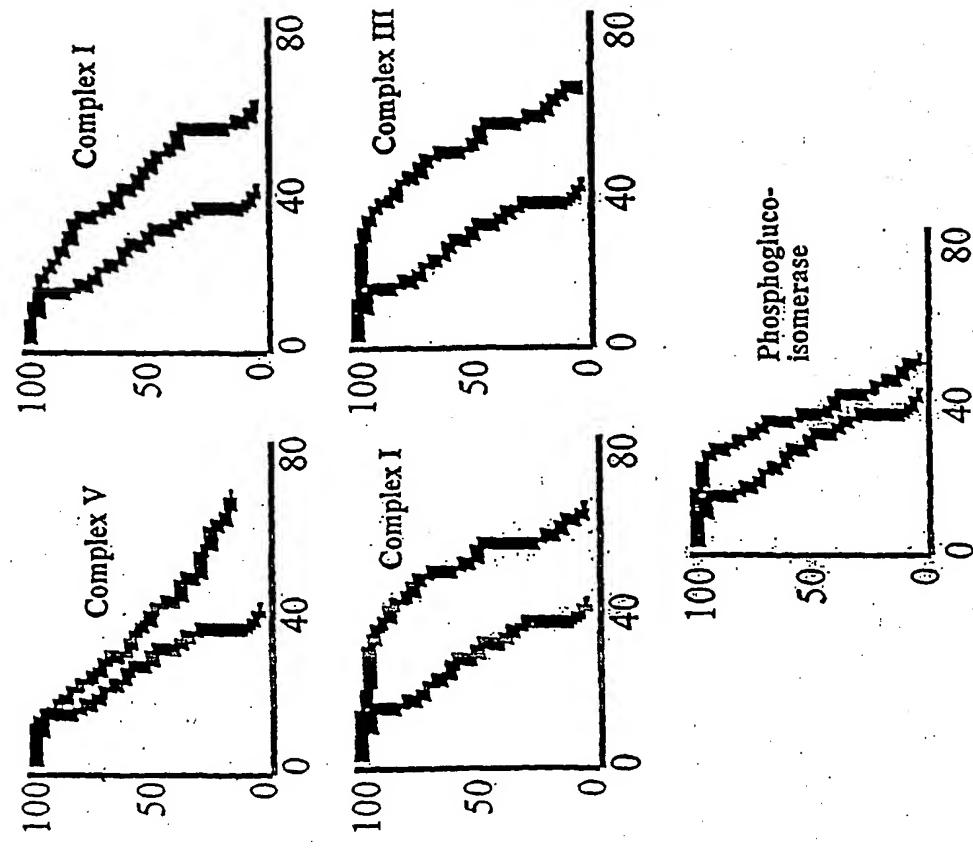


Figure 1

Figure 2

daf-2(e1370) worms treated with dsRNAs
of respiratory and glycolytic genes



daf-2(e1370) Animals

Antimycin increases life span of *C. elegans*

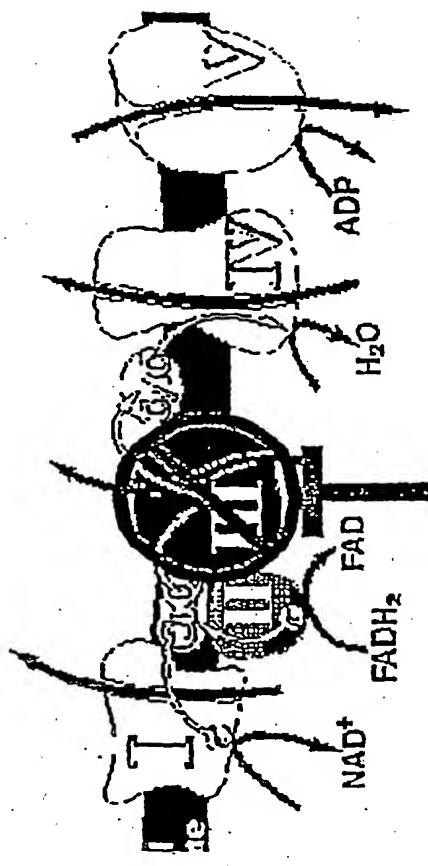
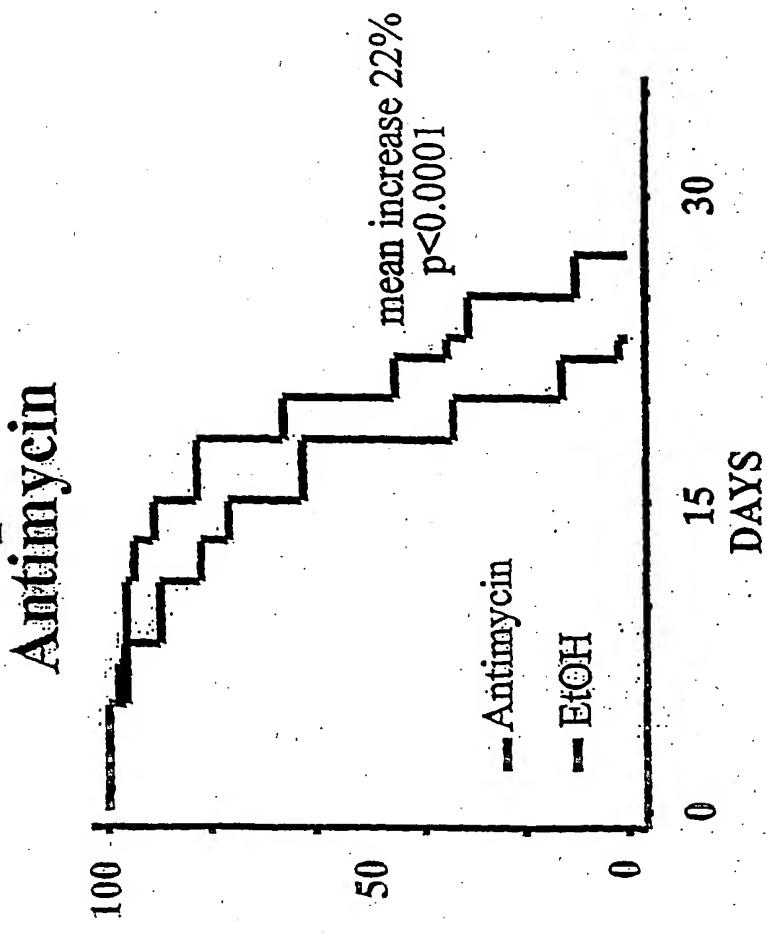


Figure 3



daf-16(mu86) mutant worms
on different dsRNA

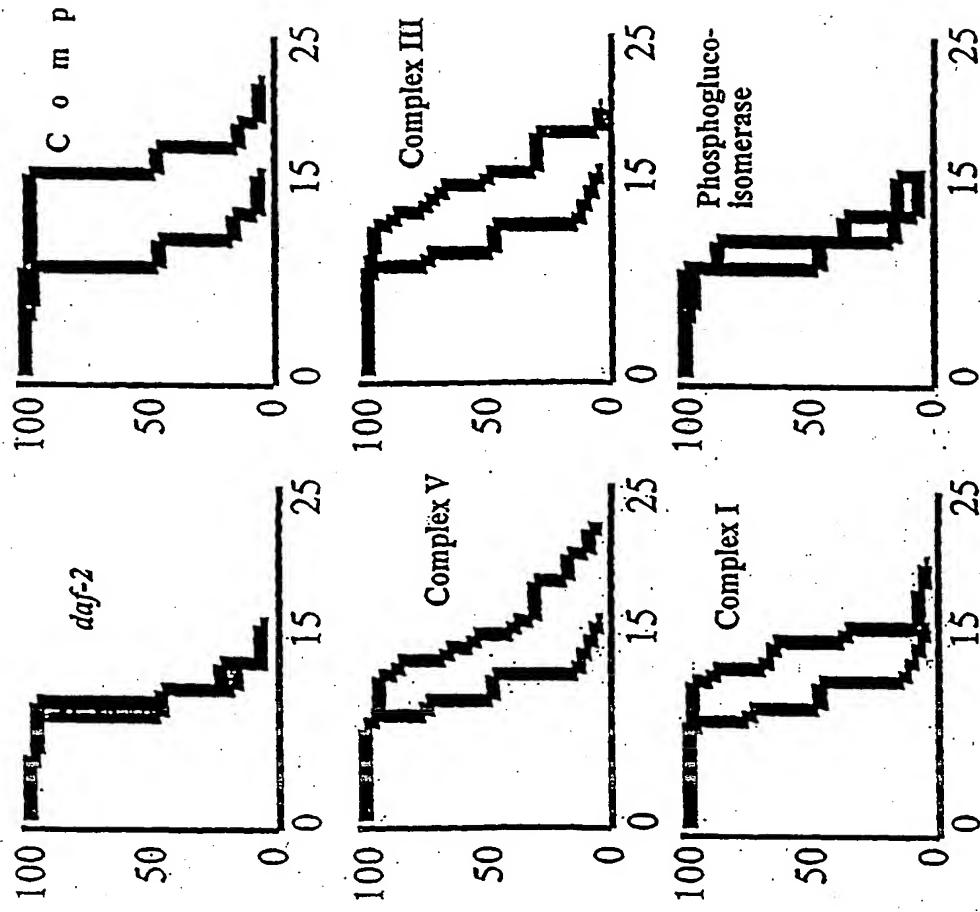


Figure 4

daf-16(mu86) Mutants

Group 1: Genes that are upregulated in *daf-2(-)* worms

Oxidative stress protection

sod-3: Manganese superoxide dismutase

ctl-1: cytosolic catalase

ctl-2: Peroxisomal catalase

mtl-1: Metallothionein; cadmium-binding

glutathione S-transferase protein family—2

©hsp-12.6, sip-1, hsp-16.1, hsp-16.11

Metabolic

acyl-CoA synthetase

alcohol dehydrogenase

Malate synthase 1/isocitrate lyase

acyl CoA dehydrogenase

hydroxyacyl-CoA dehydrogenase

dao-3: C1-tetrahydrofolate synthase

trehalase

Steroid metabolism/lipid synthesis

©cytP450 heme-thiolate protein family—3

UDP-glucuronosyltransferase protein family—4

estradiol 17 beta-dehydrogenase—2

fat-6: stearoyl-CoA delta-9 fatty acid desaturase

fat-7: stearoyl-CoA delta-9 fatty acid desaturase

fat-3: delta6-fatty acid-desaturase activity

ASAH acid ceramidase

Figure 5

Antibacterial, antifungal

Thaumatin-like antifungal—3

Bactericidal amoebapores; antibacterial agent

Pathogen-related in yeast (PRY) protein family

N-acetyl/muraminidase—2

Proteolysis

ges-1: Carboxylesterase expressed in gut cells

gcp-1: Cysteine protease expressed in the Intestine

cathepsin Z-like cysteine protease

Many unknown genes/
members of unknown families

Figure 6

hsp-12.6 RNAi reduces *daf-2* longevity

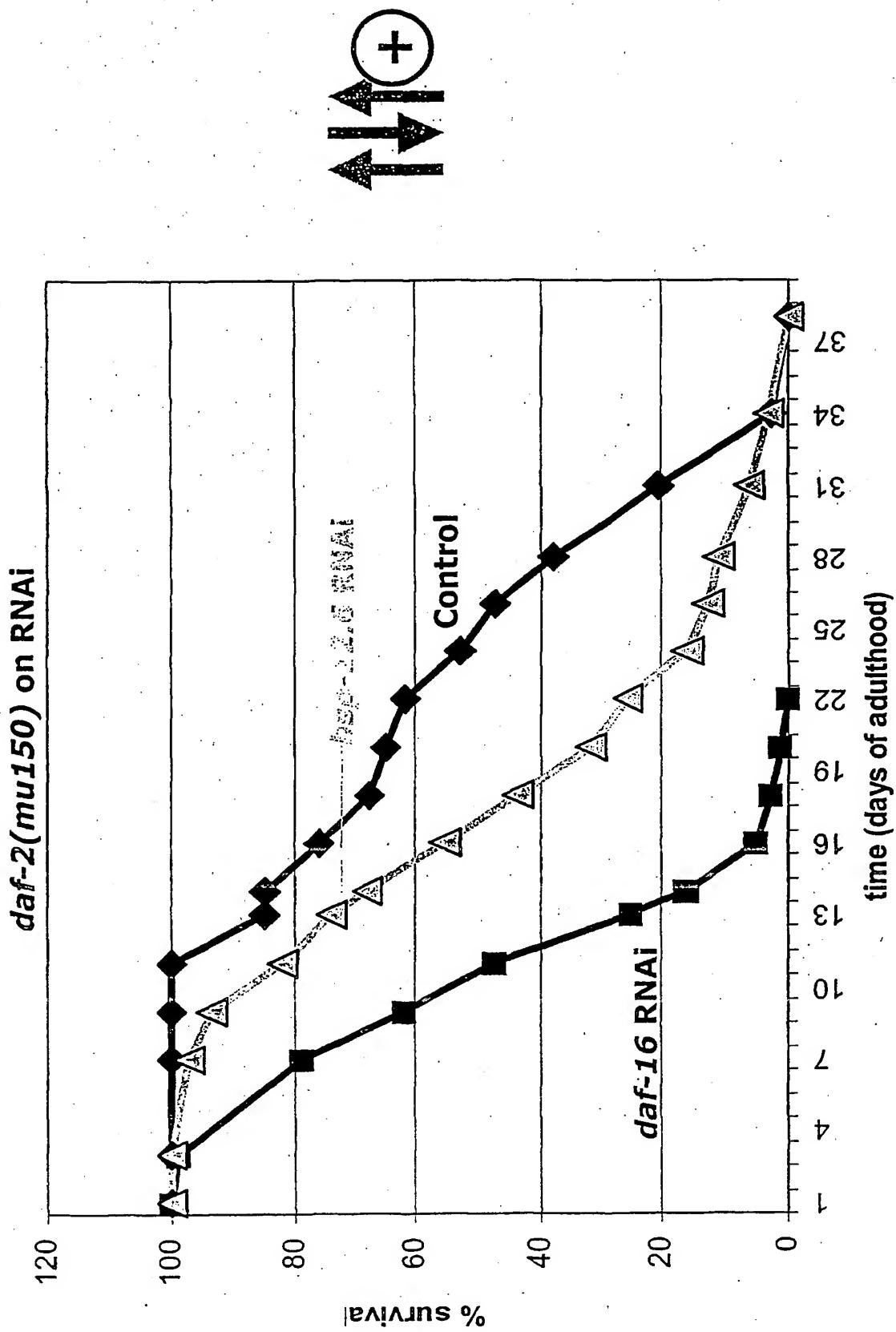


Figure 7.

...as does *mtl-1* RNAi

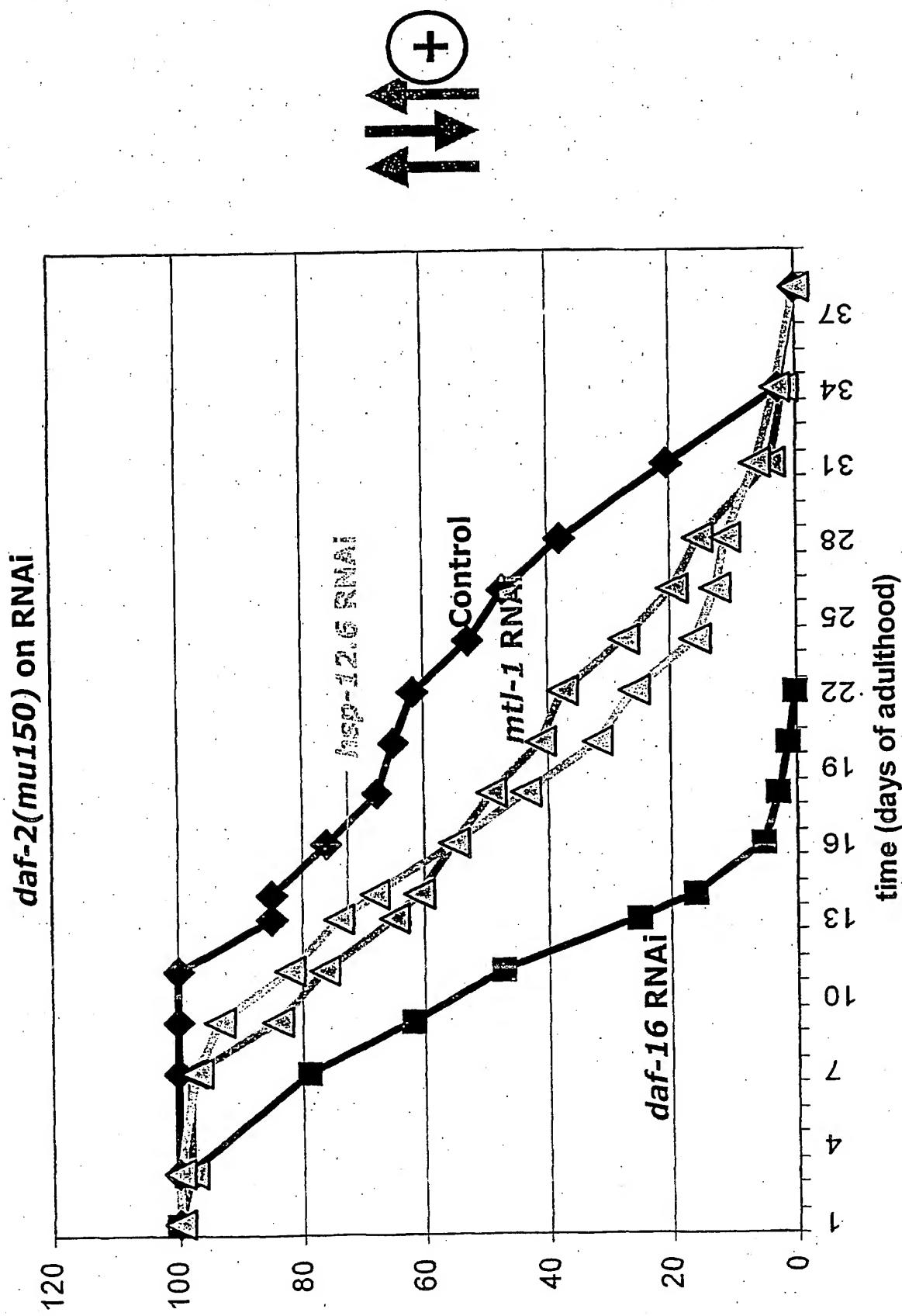


Figure 8
A cytochrome P450 shortens *daf-2* lifespan

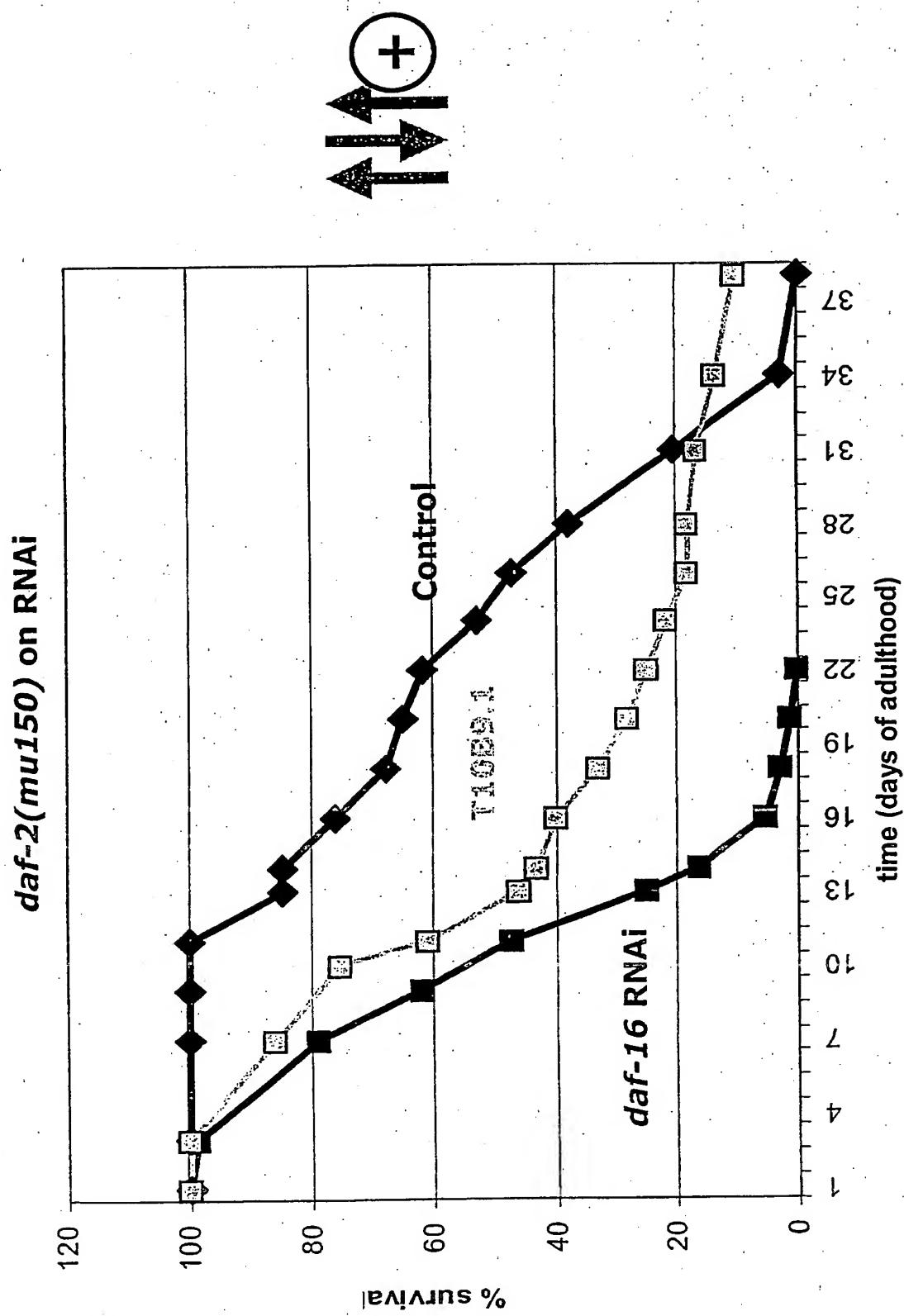


Figure 9

Group 2: Genes downregulated in *daf-2* mutants

Neuronal

- similarity to human ASMTL protein, acetylserotonin N-methyltransferase-like protein

Putative G protein-coupled receptor, no homolog found in human or *D. melanogaster*

gcy-18: Protein with a cytoplasmic receptor tyrosine kinase domain and a guanylate cyclase domain,
has strong similarity to human natriuretic peptide receptor NPR1

gcy-6: Putative guanylyl cyclase expressed in the ASEL neuron

Other (most are unidentified)

- vitellogenins

nhr-64: Member of the nuclear hormone receptor/zinc finger protein family

pep-2: Member of the proton-coupled oligopeptide transporter superfamily

mtl-2: Metallothionein-related, cadmium-binding protein

Ibp-7: Member of the fatty acid-binding protein family

aminopeptidases--many

Carboxypeptidases--many

- **ins-7:** Insulin-related protein of the type-beta subfamily

Figure 10

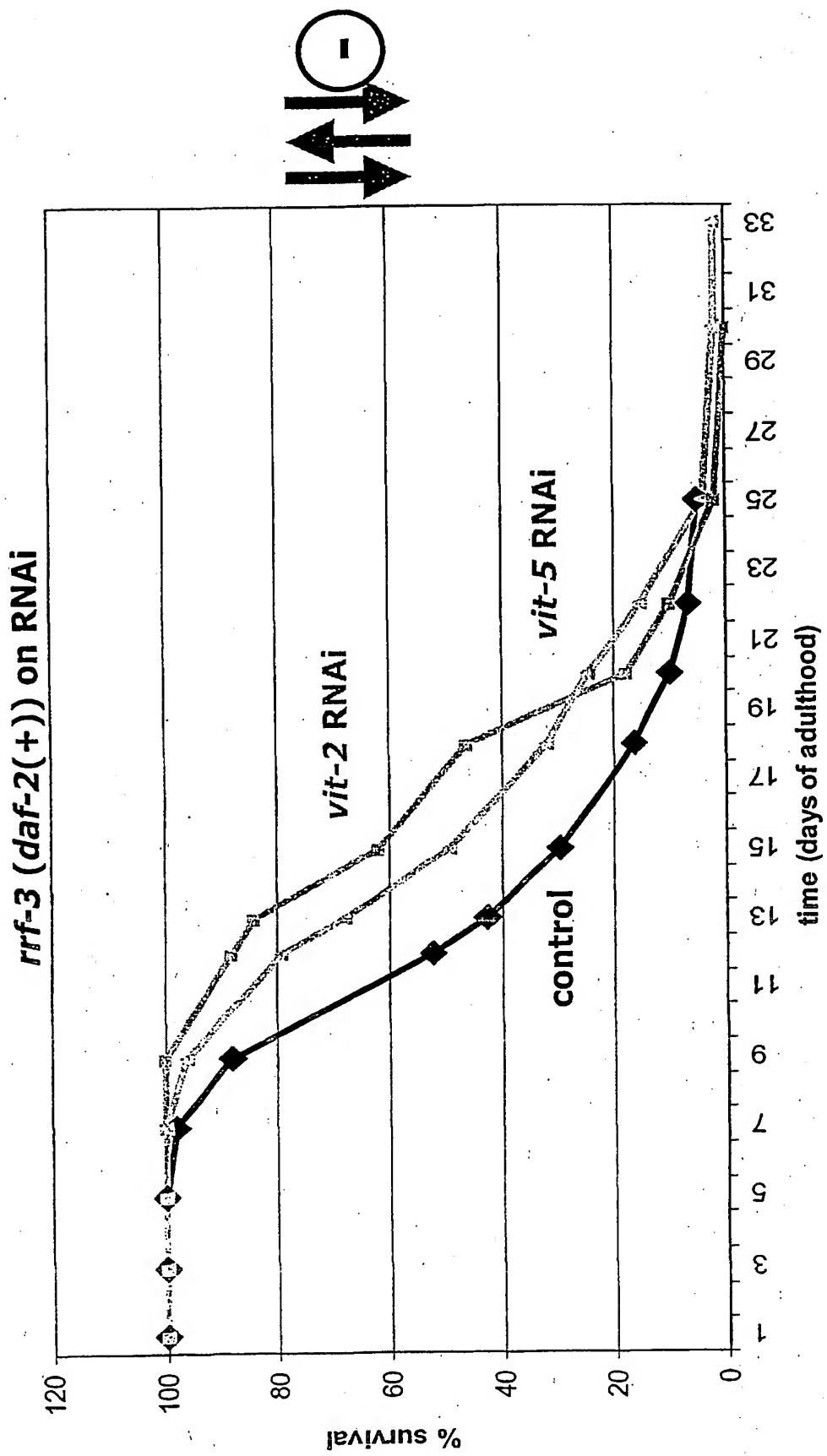
RNAi of vitellogenins increases lifespan

Figure 11

RNAi of a putative melatonin synthesis gene also prolongs lifespan

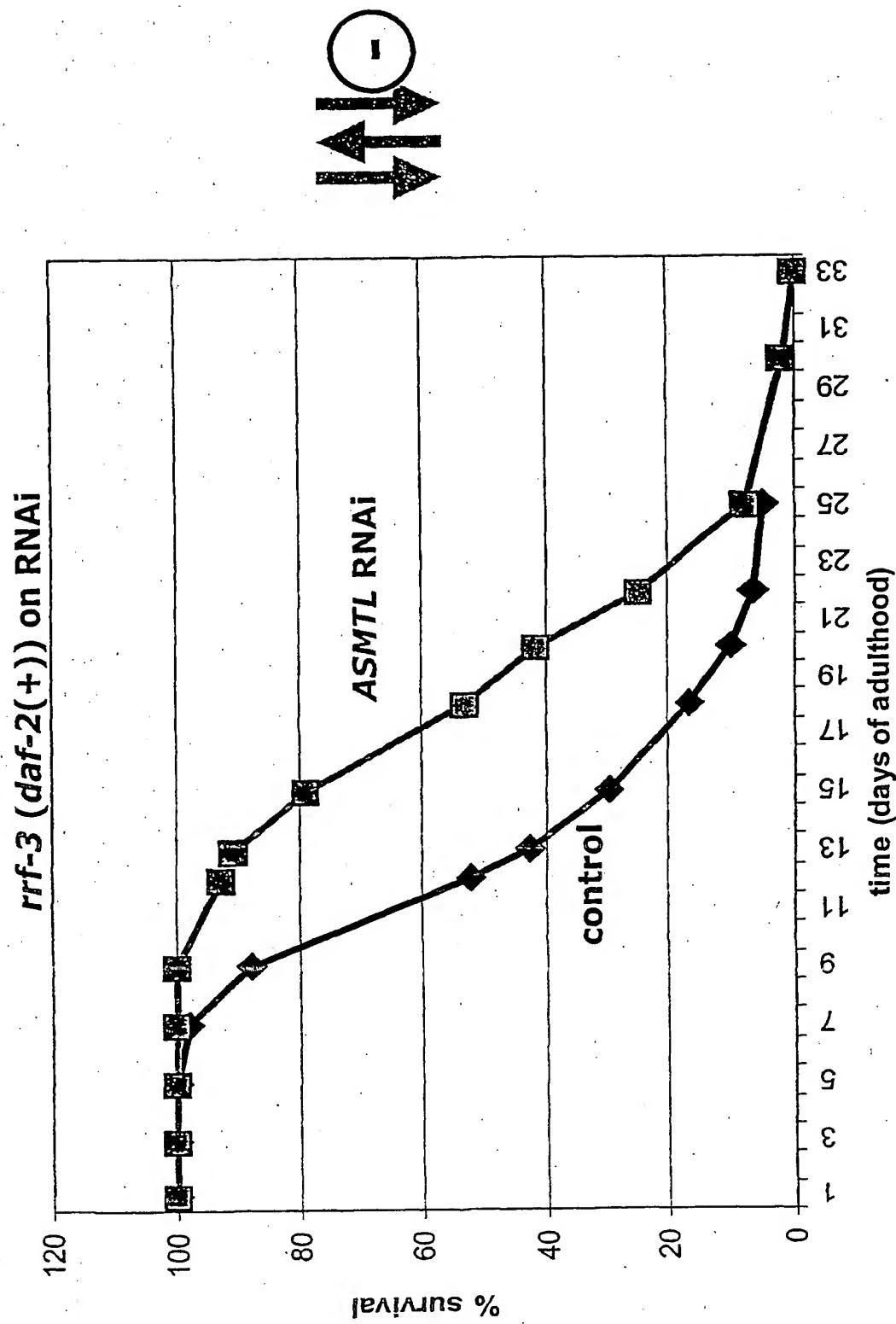
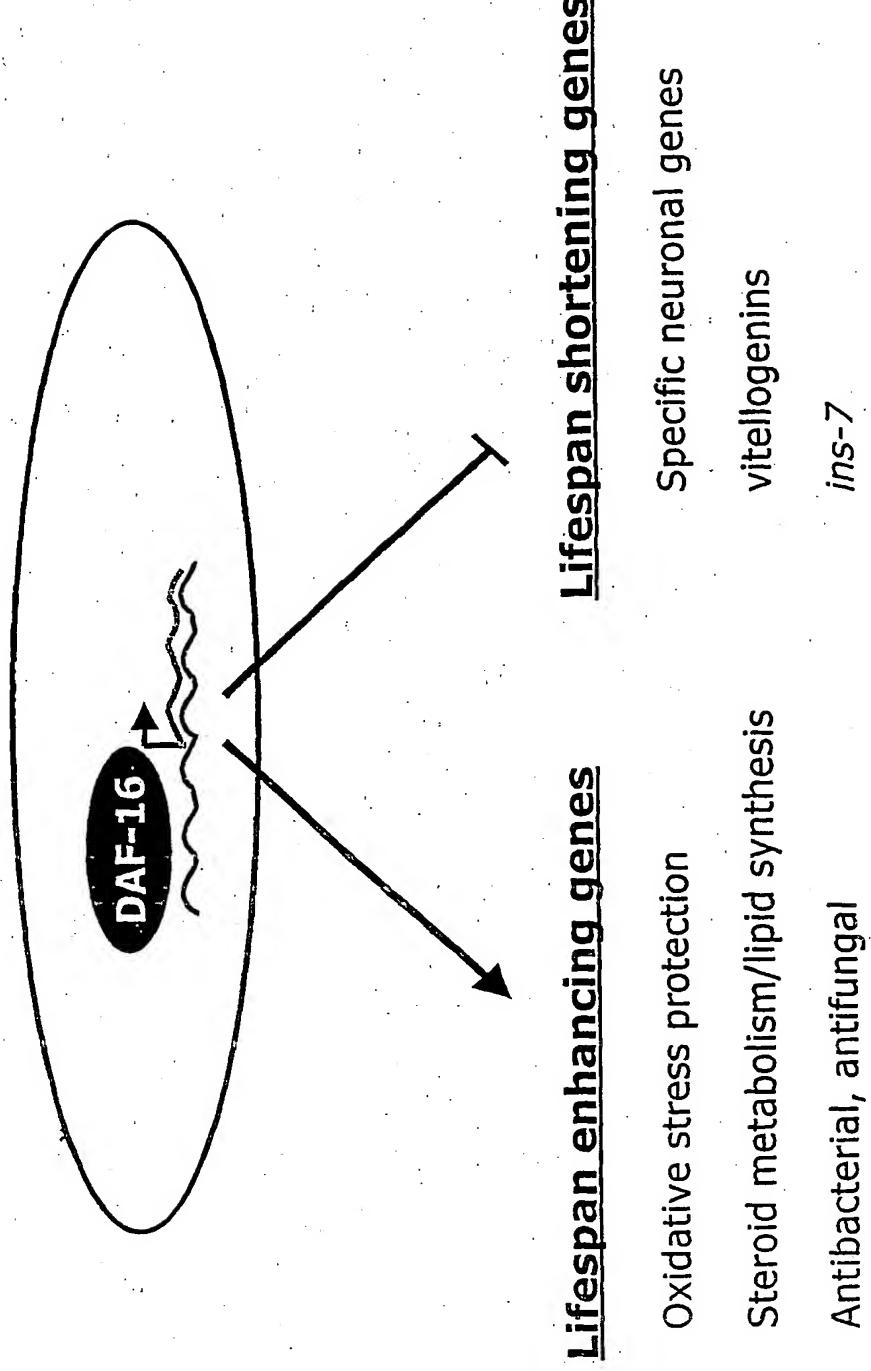


Figure 12

DAF-16 increases lifespan through a combination of induction and repression of lifespan-affecting genes



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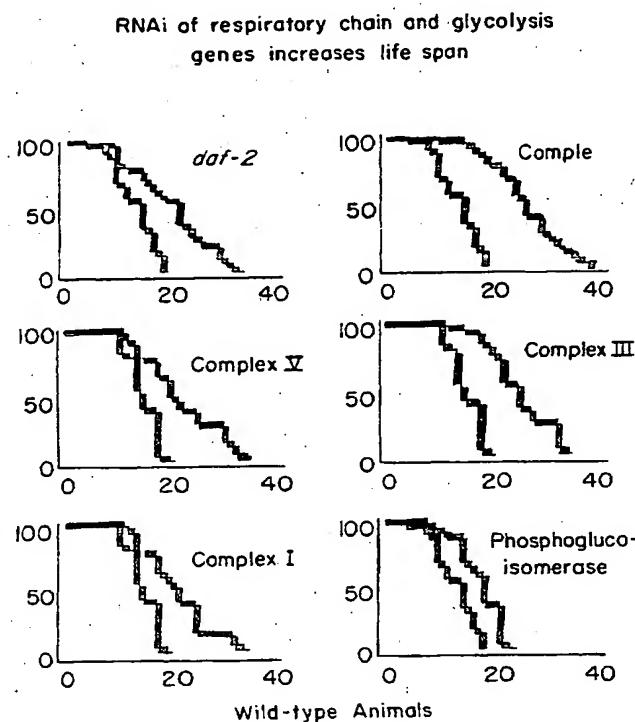
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60:373,975 18 April 2002 (18.04.2002) US(71) Applicant (*for all designated States except US*): **THE REGENTS OF THE UNIVERSITY OF CALIFORNIA**
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[Continued on next page]

(54) Title: EUKARYOTIC GENES INVOLVED IN ADULT LIFESPAN REGULATION

**WO 03/000861 A2**

expectancy, and aging. The present invention also relates to gene therapy involving lifespan associated genes.

(57) Abstract: The present invention relates to regulation of adult lifespan in eukaryotes. More particularly, the present invention is directed to methods of assaying for genes, gene products, and genes in pathways controlled by such genes and gene products, using RNAi and microarray analysis, that regulate lifespan (e.g., extend or truncate adult lifespan) in eukaryotes such as invertebrates (e.g., *C. elegans*), plants, and mammals, e.g., humans. For example, the present invention is directed to genes encoding components of the mitochondrial respiratory chain and genes encoding glycolysis enzymes, which are involved in lifespan regulation, and genes and gene products in pathways controlled by such genes. Other genes and gene products identified as regulating aging and aging pathways include a gene encoding a GTPase; a transcriptional activator; novel genes: llw-1, llw-2, llw-3, and llw-4; genes encoding cytochrome P450 proteins (involved in steroid biosynthesis); a melatonin synthesis gene; genes encoding insulin and insulin-like peptides; genes encoding heat shock factors; genes encoding catalases; stress-response genes; and metabolic genes. The invention further relates to methods for identifying and using agents, including small molecule chemical compositions, antibodies, antisense nucleic acids, and ribozymes, that regulate, e.g., enhance, adult lifespan via modulation of aging associated proteins; as well as to the use of expression profiles, markers, and compositions in diagnosis and therapy related to lifespan extension, life



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EUKARYOTIC GENES INVOLVED IN ADULT LIFESPAN REGULATION

CROSS-REFERENCES TO RELATED APPLICATIONS

5 The present application is related to USSN 60/300,577, filed June 22, 2001, USSN 60/301,052, filed June 25, 2001, and USSN 60/373,975, filed April 18, 2002, each herein incorporated by reference in their entirety.

STATEMENT AS TO RIGHTS TO INVENTIONS MADE UNDER 10 FEDERALLY SPONSORED RESEARCH AND DEVELOPMENT

This invention was made with government support under Grant No. NIH AG11816, awarded by the NIH. The government has certain rights in this invention.

FIELD OF THE INVENTION

15 The present invention relates to regulation of lifespan in eukaryotes. More particularly, one aspect of the present invention is directed to methods of assaying for genes, gene products, and genes in pathways controlled by such genes and gene products, using RNAi and microarray analysis, that regulate lifespan (e.g., extend or truncate adult lifespan) in eukaryotes such as invertebrates (e.g., *C. elegans*), plants, and mammals, e.g., humans.
20 For example, one aspect of the present invention is directed to genes, in particular human genes, encoding components of the mitochondrial respiratory chain and genes encoding glycolysis enzymes, which are involved in lifespan regulation, and genes and gene products in pathways controlled by such genes. Other genes and gene products identified as regulating aging and aging pathways include a gene encoding a GTPase; a transcriptional activator;
25 novel genes: *llw-1*, *llw-2*, *llw-3*, and *llw-4*; genes encoding cytochrome P450 proteins (involved in steroid biosynthesis); a melatonin synthesis gene; genes encoding insulin and insulin-like peptides; genes encoding heat shock factors; genes encoding catalases; stress-response genes; and metabolic genes. The invention further relates to methods for identifying and using agents, including small molecule chemical compositions, antibodies, antisense
30 nucleic acids, and ribozymes, that regulate, e.g., enhance, adult lifespan via modulation of aging associated proteins; as well as to the use of expression profiles, markers, and

compositions in diagnosis and therapy related to lifespan extension, life expectancy, and aging. The present invention also relates to gene therapy involving lifespan associated genes.

BACKGROUND OF THE INVENTION

Previously, classic genetic screens have been used to identify genes involved in the *C. elegans* development. In one example, inhibition of mitochondrial respiratory chain genes such as NADH ubiquinone oxidoreductase and ATP synthase in *C. elegans* larva was found to impair larval development and cause arrest in the third larval stage (see, e.g., Tsang et al., *JBC* 276:33240-33246 (2001)). In other examples, classical genetic screens have been used to identify *C. elegans* genes involved in a variety of processes, including dauer formation, and embryonic development. Some of these genes, for example the *daf-2* and *daf-16* genes, have been implicated in the regulation of lifespan see, e.g., Kenyon et al., *Nature* 366:461-464 (1993); Morris et al., *Nature* 382:536-539 (1996); Kimura et al., *Science* 277:942-946 (1997); Paradis et al., *Genes Dev.* 12:2488-2498 (1998); Paradis et al., *Genes Dev.* 13:1438-1452 (1999); Off & Ruvkun, *Mol. Cell* 2:886-893 (1998); Guarente & Kenyon, *Nature* 408:255-262 (2000); Ogg et al., *Nature* 389:994-999 (1997); and Lin et al., *Science* 278:1319-1322 (1997)).

Classical genetic screens are frequently time consuming, both in identification of interesting mutants and in cloning a gene associated with a mutation. Classical genetic screens can include labor intensive backcrosses to eliminate mutations unlinked to the phenotype of interest. Classical genetic screens also may require the extra step of cloning the gene of interest, by complementation of the mutation.

Many different genes likely regulate the process of aging in eukaryotes and their identification will aid in understanding the process. Regulation of biological processes is frequently conserved between divergent organisms. For example, cell cycle proteins and their mechanism of action are conserved between organisms as divergent as yeast and humans. Thus, regulatory mechanisms identified in a genetically tractable organism can be used to predict and identify homologous genes and gene products that regulate similar biological processes in higher eukaryotes. However, even in genetically tractable organisms, such as *C. elegans*, classical genetic methods are frequently labor intensive and cumbersome for identification of interesting mutants and for isolation of a gene of interest. The present invention solves these and other problems.

SUMMARY OF THE INVENTION

The present invention provides, for the first time, methods of assaying for aging associated genes and proteins using RNAi, as well as aging associated genes and proteins so identified. Double-stranded RNA libraries are administered to a test organism or cell, e.g., *C. elegans*. For administration to *C. elegans*, the RNAi library can be expressed in a bacterial cell and fed to the *C. elegans*. Age-associated mutants (those that live longer or age faster) are identified by measuring lifespan increase or decrease, or an age-associated parameter such as stress resistance or a Nomarski analysis indicia, e.g., yolk accumulation, loss of ability to chew and expel (distended oral and anal cavities), necrotic cavities in tissue, curdled appearing tissue, and germ cell appearance (graininess, large, well separated nuclei, fewer nuclei, and cavities).

Other methods can be used to identify age-associated genes and gene products, including microarray analysis, database profiling with known age-associated genes, mammalian complementation assays, yeast two hybrid assays, immunoprecipitation, etc.

The use of dsRNA has identified a number of genes and gene products which, when inhibited by dsRNA, results in longer lifespans, e.g., phosphoglucose isomerase, cytochrome C1, NADH oxidoreductase, ATP synthase, cytochrome C oxidase, a GTPase, and four novel genes, *llw1-4* (see also Tables 5 and 6). Thus, compounds that inhibit such genes and gene products, as well as genes in a pathway controlled by such genes and gene products, would be useful for increasing lifespan and modulating the aging process in eukaryotes, e.g., plants, mammals, humans. The genes identified herein include any mammalian or human homologs thereof.

In addition, the use of dsRNA has identified genes and gene products which, when inhibited by dsRNA, results in a shorter lifespan, e.g., heat shock protein or heat shock factor (HSF) (see also Table 5 and Table 6). Overexpression of HSF has also been shown to increase lifespan in *C. elegans*. Thus, compounds that activate heat shock proteins or genes such as HSF-1 or HSF targets, or genes or gene products controlled by HSF, or genes that respond to stress and activate HSF would be useful for increasing lifespan and modulating the aging process in eukaryotes; e.g., plants, mammals, humans. In one example, compounds such as zinc finger proteins (naturally occurring or recombinant) can be used to bind to HSF binding regions on genes and alter expression of HSF-controlled genes (see, e.g., U.S. 5,789,538 and 6,242,568).

Microarrays were used to analyze gene expression profiles in *daf-2* and *daf-16* mutants and identify genes and gene products involved in lifespan regulation. The expression

of a number of genes varied (by over- or under-expression) in long-lived (*daf-2*) or short-lived (*daf-16* and *daf-16; daf-2*) *C. elegans* mutants (see Table 5). The activity of genes identified using microarray analysis was then modulated using RNAi (Table 6). The genes thus identified include hormones that activate the *daf-2* pathway, several encoding cytochrome P450 proteins (involved in steroid biosynthesis), the melatonin synthesis gene, insulin and insulin-like peptides, heat shock factors, catalases, stress-response genes, and metabolic genes. These genes and gene products, and genes and gene products controlled by these genes (e.g., steroid hormones, melatonin) are therefore useful for developing drugs to regulate aging in eukaryotes, e.g., plants, mammals, humans. In addition, these genes can be used as markers for the insulin/IGF system activity, as markers for the aging process, and as markers that indicate the likely longevity of an individual.

In one aspect of the invention, nucleic acids from *C. elegans* and corresponding mammalian genes (e.g., human) encoding glycolysis proteins, e.g., phosphoglucose isomerase, and mitochondrial respiratory chain proteins, e.g., cytochrome C1 component of complex III (CYC1), NADH oxidoreductase (NUO2), ATP synthase (ATP3, a member of the ATP synthase delta family), and cytochrome C oxidase (CCO1), are provided, as are nucleic acids from *C. elegans* and corresponding mammalian genes encoding a GTPase associated with aging; a transcriptional activator, heat shock factors, e.g., HSF-1; novel proteins LLW-1, LLW-2, LLW-3, and LLW-4; cytochrome P450 proteins, a melatonin synthesis gene, insulin and insulin-like peptides, heat shock factors, catalases, stress-response genes, and other genes listed in Tables 5 and 6. In another aspect, the present invention provides nucleic acids, such as probes, antisense oligonucleotides, and ribozymes, that hybridize to glycolysis genes, e.g., phosphoglucose isomerase (GPI-1); mitochondrial respiratory chain genes, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and cytochrome C oxidase; a GTPase associated with aging; a transcriptional activator, heat shock factors, e.g., HSF-1; novel genes *llw-1*, *llw-2*, *llw-3*, and *llw-4*; cytochrome P450 proteins, a melatonin synthesis gene, insulin and insulin-like peptides, heat shock factors, catalases, stress-response genes, and other genes listed in Tables 5 and 6.

In another aspect, the invention provides expression vectors and host cells comprising nucleic acids encoding glycolysis proteins, e.g., phosphoglucose isomerase; mitochondrial respiratory chain proteins, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and cytochrome C oxidase; a GTPase associated with aging; a transcriptional activator, heat shock factors, e.g., HSF-1; novel genes *llw-1*, *llw-2*, *llw-3*, and *llw-4*; cytochrome P450 proteins, a melatonin synthesis gene, insulin and insulin-like peptides, heat

shock factors, catalases, stress-response genes, and other genes listed in Tables 5 and 6. In another aspect, the present invention provides glycolysis proteins, e.g., phosphoglucose isomerase; mitochondrial respiratory chain proteins, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and cytochrome C oxidase; a GTPase associated with aging; a transcriptional activator, the heat shock factor; novel proteins LLW-1, LLW-2, , LLW-3, 5 LLW-4; cytochrome P450 proteins, a melatonin synthesis protein, insulin and insulin-like peptides, heat shock factors, catalases, stress-response proteins, and other gene products listed in Tables 5 and 6 and antibodies thereto.

In another embodiment, the invention provides heterologous constructs 10 comprising an age-associated gene as described herein, and a heterologous sequence such as a regulatory region, a reporter gene, a purification tag, e.g., for production of a fusion protein, for purification of a gene product, for more efficient expression of the gene or gene product, or for regulated expression of the gene.

In one embodiment, methods known to those of skill in the art such as RT-PCR, northern, Southern analysis, cDNA and genomic library cloning, etc. can be used to 15 identify eukaryotic orthologs, e.g., invertebrate, vertebrate, plant, mammalian, and human orthologs, of the age-associated proteins provided herein. In another embodiment, computer sequence analysis can be used to identify orthologs. Such methods optionally include the step of assessing an age associated parameter in a cell in which the suspected ortholog is 20 perturbed.

In one embodiment, endogenous or recombinant gene products of the age 25 associated genes described herein are purified using the methods described herein, to at least about 50% purity, preferably 60%, 70%, 80%, 90% or higher purity. In another embodiment, the present invention provides a reaction mixture comprising an age-associated protein and another component such as a test compound, an antibody, a peptide, etc.

In another aspect, the present invention provides a method for identifying a compound that modulates adult aging, the method comprising the steps of: (i) contacting the compound with a glycolysis protein, e.g., phosphoglucose isomerase, or a mitochondrial respiratory chain protein, e.g., cytochrome C1, NADH oxidoreductase, ATP synthase, and 30 cytochrome C oxidase or a GTPase associated with aging; a novel proteins LLW-1, LLW-2, LLW-3, LLW-4; and (ii) determining the functional effect, e.g., lifespan effect or another age-associated parameter of the compound upon the polypeptide.

In another aspect, the present invention provides a method for identifying a compound that modulates adult aging, the method comprising the steps of: (i) contacting the

compound with protein encoded by a gene listed in Table 5 or 6; and (ii) determining the functional effect, e.g., lifespan effect or another age-associated parameter of the compound upon the polypeptide.

In another aspect, the present invention provides a method for identifying a compound that modulates adult aging, the method comprising the steps of: (i) contacting the compound with a heat shock factor and (ii) determining the functional effect, e.g., lifespan effect or another age-associated parameter, of the compound upon the polypeptide.

5 In one embodiment, the functional effect is a physical effect or a chemical effect. In another embodiment, the functional effect is a phenotypic effect. In one embodiment, the polypeptide is expressed in a eukaryotic host or host cell, e.g., *C. elegans*. In another embodiment, the functional effect is determined by measuring longevity, average lifespan, or mean lifespan of an organism contacted with a compound. In one embodiment, the functional effect is determined by measuring enzymatic activity. In one embodiment, the functional effect is determined by measuring transcriptional activation. In one embodiment, the organism is *C. elegans*. In another embodiment, the organism is mammalian host or cell, e.g., a mouse, a rat, a guinea pig, a monkey, or a human.

10 In another embodiment, compounds that modulate aging are identified using computer programs that model age-associated protein structure and determining compounds that bind or interact with the modeled protein. Optionally, the effect of the compound can be validated by examining its effect on a cell or organism expressing the modeled age-associated protein.

15 In another embodiment, the method comprises providing a sequence comprising an age-associated protein, altering the sequence, e.g., by mutagenesis, and assaying the protein encoded by the altered sequence.

20 In another aspect, the present invention provides a method of modulating lifespan in a subject, the method comprising the step of contacting the subject with an therapeutically effective amount of a compound identified using the methods described herein, e.g., a compound such as antimycin. In one embodiment, the subject is *C. elegans*. In another embodiment, the subject is a mammalian subject, e.g., a mouse, a rat, a guinea pig, a monkey, or a human. In one embodiment, the subject is a plant. In one embodiment, the compound is antimycin or an analog thereof.

25 In another aspect, the present invention provides a method of detecting the presence of a lifepan associated protein described herein, and the genes encoding such proteins in eukaryotic tissue, the method comprising the steps of: (i) isolating a biological

sample; (ii) contacting the biological sample with a specific reagent that selectively associates with the protein of choice; and, (iii) detecting the level of specific reagent that selectively associates with the sample.

In one embodiment, the specific reagent is selected from the group consisting
5 of: antibodies, oligonucleotide primers, and nucleic acid probes.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows RNAi of respiratory chain and glycolysis genes increasing lifespan.

10 Figure 2 shows *daf-2(e1370)* worms treated with dsRNAs of respiratory and glycolytic genes.

Figure 3 shows antimycin increases lifespan.

Figure 4 shows *daf-16(mu86)* mutant worms treated with dsRNAs of respiratory and glycolytic genes.

15 Figure 5 shows certain genes identified using microarray analysis that are upregulated in *daf-2(-)* worms.

Figure 6 shows that hsp-12.6 RNAi reduces *daf-2* longevity.

Figure 7 shows that mtl-1 RNAi reduces *daf-2* longevity.

Figure 8 shows that cytochrome P450 shortens *daf-2* lifespan.

20 Figure 9 shows certain genes identified using microarray analysis that are downregulated in *daf-2* mutants.

Figure 10 shows that RNAi of vitellogenins increases lifespan.

Figure 11 shows that RNAi of ASMLT (melatonin synthesis gene) prolongs lifespan.

25 Figure 12 shows certain genes that modulate lifespan.

DETAILED DESCRIPTION OF THE INVENTION

INTRODUCTION

Double-stranded RNA-mediated interference (RNAi) provides a sequence specific mechanism for inhibiting gene expression in *C. elegans* (see, e.g., Fire *et al.*, *Nature* 391:806-811 (1998) and WO 99/32619)). This technique is also useful for functional genomics analysis of *C. elegans* genes (see, e.g., Fraser *et al.*, *Nature* 408:325-330 (2000); Kamath *et al.*, *Genome Biol.* 2:RESEARCH0002 (2000)).

Surprisingly, RNAi can be used to identify genes and gene products involved in lifespan and aging mechanisms in adults, by feeding bacteria expressing a dsRNA library over long-term time periods to *C. elegans* and measuring, e.g., mean and median lifespan increase or decrease in adults. Such assays can be used, in addition, to determine if two or 5 more genes function together in a similar aging pathway, to determine if gene function is cell autonomous, and to determine if drug compounds known to alter the aging process function in the same or different pathways. Microarray assays can also be used to identify genes and gene products involved in lifespan and aging (see, e.g., Table 5 and Table 6).

Microarray and RNAi assays are also useful in combination, e.g., by 10 identifying a gene or gene product involved in lifespan using RNAi and then examining its expression pattern in old and young adults using microarray analysis, or by identifying a differentially expressed gene or gene product involved in lifespan using microarray analysis and then examining the effects of gene or gene product inhibition using RNAi. Microarray analysis can also be performed on animals treated with RNAi of genes identified using 15 microarray analysis.

Using the technique of double-stranded RNA inhibition (RNAi), two types of metabolic genes and gene products have been found to regulate the adult lifespan of a model eukaryote, *C. elegans*. Inhibition of genes and gene products that function in the mitochondrial respiratory chain, including genes encoding cytochrome C1, cytochrome C oxidase and ATP synthetase, lengthen lifespan, as does inhibition of a gene that functions in glycolysis, glucose phosphate isomerase. However, these perturbations produce different 20 phenotypes. Inhibition of respiratory chain components cause a Clk (clock) phenotype, including slow growth to adulthood, and, in adults, slow movement, and decreased pumping and defecation rates. In contrast, inhibition of glucose phosphate isomerase extends adult 25 lifespan without producing a Clk phenotype. Together these findings indicate that at least two distinct pathways can regulate lifespan in response to changes in metabolic gene activities in eukaryotes. These RNAi-mediated phenotypes are examples of "reverse phenotypes," i.e., suppression or inhibition of the gene lengthens lifespan. For RNAi interventions that decrease lifespan, restoring gene expression should restore a normal 30 lifespan. Therefore, under- and over-expression of lifespan genes, gene products and pathways, as well as activation or inhibition of lifespan gene, gene products, and pathways, can be used to modulate aging in a subject.

The present invention therefore provides nucleic acids from *C. elegans* encoding cytochrome C1 component of complex III, gene C54G4.8 (CYC1, SEQ ID NO:1,

Accession No. CAA99820.1, which is 50% identical to human ortholog CYC1); NADH oxidoreductase, gene T10E9.7 (NUO2, SEQ ID NO:2, Accession No. AAB522474.1, which is 56% identical to human ortholog NDUFS3, Accession No. NM_004551); ATP synthase (delta family), gene F27C1.7 (ATP3, SEQ ID NO:3, Accession No. AAB37654.1, 43% identical to human ortholog ATP50); and cytochrome C oxidase, gene F26E4.9 (CCO1, SEQ ID NO:4, Accession No. CAB03002.1, 35% identical to human ortholog COX5B, Accession No. NM_001862), which are protein components of the mitochondrial respiratory chain. The present invention also provides nucleic acids from *C. elegans* encoding glucose phosphate isomerase, gene Y87G2A.8 (GPI-1, SEQ ID NO:5, Accession No. CAB60430.1, 68% identical to human ortholog GPI, Accession No. NM_000175). The present invention also provides gene and gene products shown in Tables 5 and 6, and human homologs thereof. The present invention also provides homologs of these conserved proteins that are found in mammals, such as humans, and which are known to those of skill in the art. The invention therefore provides methods of screening for compounds, e.g., small molecules, antibodies, antisense molecules, and ribozyme, that are capable of modulating lifespan in adult eukaryotes, in particular, in mammals, e.g., for lifespan enhancement and treatment of premature aging.

Furthermore, it was discovered that administration of antimycin, a mitochondrial respiration inhibitor, substantially increased lifespan in the model *C. elegans*.

The present invention includes other genes and gene products that regulate the aging process in *C. elegans*. One such gene, encodes a GTPase, gene T23H2.5; (SEQ ID NO:6, Accession No. AAC48200.1). Inhibition of GTPase results in a longer lifespan.

Four other genes and gene products that regulate aging in *C. elegans* are also provided: *llw-1*, gene Y54G11A.8 (SEQ ID NO:7, Accession No. CAA22452); *llw-2*, gene F59E12.10 (SEQ ID NO:8, Accession No. AAB54251); and *llw-3*, gene Y48E1B.1 (SEQ ID NO:9, Accession No. CAB07688). Also included is *llw4*, gene F45H10.4 (SEQ ID NO:10; Accession No. CAB04386). Inhibition of these genes results in a Long-Lived Worm (*llw*) phenotype.

Also using the technique of double stranded RNA inhibition, a gene encoding a heat shock factor (HSF) or heat shock protein (HSP) was found to regulate aging in *C. elegans*. Inhibition of the HSF gene and gene product resulted in worms that display the objective characteristic of aging prematurely (described below) and have short lifespans compared to control worms. HSF is a transcriptional activator of stress related genes. The present invention provide nucleic acids from *C. elegans* encoding HSF-1, gene Y53C10A.12

(SEQ ID NO:11, Accession No. CAA22146). As described above, for RNAi treatments that decrease lifespan, restoring gene expression should restore a normal lifespan. Furthermore, overexpression of this gene in *C. elegans* extends lifespan.

It has been discovered that old worms have a number of characteristic features 5 that are highly recognizable and diagnostic when the animals are viewed with Nomarski optics. These characteristics allow for a determination of whether or not a worm is aging prematurely, aging normally, aging slowly, or not aging at all, e.g., has a mutation that causes a sick rather than an aging phenotype. Thus, these objective characteristics can be used as an assay, in combination with RNAi, for genes, gene products, and drugs involved in lifespan 10 regulation. The present invention therefore also provides genes, identified using RNAi, that regulate the aging process in adult eukaryotes.

As described above, the present invention provides a method of using RNAi to identify genes and gene products involved in lifespan regulation, by screening, e.g., for increased or decreased mean and median lifespan. The present invention also demonstrates 15 that Nomarski microscopy provides a powerful way to monitor the aging process in cells and tissues of *C. elegans*. This technique permits an objective of the quality of cells and tissues that make up most of the body mass. It was found that the tissues of aging animals have a very characteristic appearance, using a number of objective criteria, e.g., yolk accumulation, loss of ability to chew and expel (distended oral and anal cavities), bacterial packing in the 20 intestine (constipation), necrotic cavities in tissue, curdled appearing tissue, and germ cell appearance (graininess, large, well separated nuclei, fewer nuclei, and cavities). These characteristics allow for a determination of whether or not a worm is aging prematurely (progeria), aging normally, aging slowly, or not aging at all, e.g., has a mutation that causes a sick rather than an aging phenotype. Thus, these objective characteristics can be used, as an 25 assay for genes and drugs involved in lifespan regulation. In one embodiment, the characteristics are used to evaluate an organism or cell that is treated with RNAi. In another embodiment, the characteristics are used to evaluate an organism or cell that is genetically mutated. Using the technique of double stranded RNA inhibition, a gene encoding Heat Shock Factor (HSF) was found to prevent premature aging in *C. elegans*.

In one aspect, the invention features a method that includes: (1) providing a 30 library of nucleic acids, each nucleic acid of the library comprising a segment of a gene of an organism; and (2) for each member of the library, (a) generating double-stranded RNA (dsRNA) from the respective member of the library, (b) providing the dsRNA to a cell or one or more cells of an organism to provide a dsRNA treated cell or a dsRNA treated organism,

and (b) monitoring an age-associated parameter of the dsRNA treated cell or the dsRNA treated organism. Exemplary dsRNA-treated organisms include a nematode, *C. elegans*, an organism other than a nematode, *Drosophila*, zebrafish, and a mammal, e.g., a mouse. The providing of dsRNA can include feeding the organism the dsRNA or a bacterium that expresses the dsRNA. Exemplary dsRNA-treated cells include a *Drosophila* cell or a mammalian cell, e.g., a murine, canine, bovine, primate, or human cell. The cell can be culture *in vitro*. The age-associated parameter can be any age-associated parameter, e.g., a parameter described herein such as lifespan.

The method can be repeated for a cohort of dsRNA treated organisms and the age-associated parameter can include a survival curve for the cohort. The method can further include, for each member of the library indicated as affecting lifespan of the organism, evaluating abundance of an RNA or a protein corresponding to indicated library member or an RNA or protein corresponding to a gene listed in Tables 5 and 6. The abundance can be evaluated, for example, in a defined set of cells or in organisms of defined age. The abundance can be evaluated using an array, e.g., a nucleic acid microarray. The method can include other features described herein.

In another aspect, the invention features a method that includes: evaluating a relationship between presence or abundance for each species of a plurality of RNA or protein species with respect to age of a cell or organism; producing or delivering a double-stranded RNA to a cell or one or more cells of an organism, the RNA corresponding to a species whose presence or abundance is correlated with age; and monitoring an age-associated parameter of the cell or the one or more cells or the organism. Evaluating the relationship can include transcriptional profiling using a nucleic acid array. The plurality of RNA or protein species can include one or more species corresponding to a gene listed in Tables 5 and 6. The plurality can include at least 10, 20, 40, 50, 60, 70% or 100% of the genes listed in Tables 5 and 6. In one embodiment, the organism is an invertebrate. In another embodiment, the organism is a vertebrate, e.g., a mammal. Similarly, a cell can be a cell of a vertebrate (e.g., a mammal, e.g., a human) or an invertebrate, e.g., a nematode.

In another aspect, the invention features a method that includes: contacting a test compound to a living or biochemical system that includes a target protein selected from the group consisting of: a respiratory chain component, a heat shock promoter activator (e.g., an HSF protein or is an artificial chimeric transcription factor), LLW-1, LLW-2, LLW-3, LLW-4, a small GTPase, a protein encoded by a gene listed in Table 5 and 6, and mammalian (e.g., human) homologues thereof; and evaluating a property associated with the target

protein. The method can be used, e.g., to evaluating a test compound, e.g., for an effect on lifespan or a lifespan-related process. In one embodiment, the target protein is a *C. elegans* protein, e.g., a protein that includes SEQ ID NO:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or 11. In another embodiment, the target protein is a mammalian protein.

5 The living or biochemical system can be a mammalian or non-mammalian system. In one embodiment, the system includes a cell extract, e.g., a lysate or fraction of a cell, e.g., a membrane preparation, a cytoplasmic preparation, or a partially or completely purified preparation. In another embodiment, the system includes isolated mitochondria, e.g., a cell extract or fraction enriched in mitochondria. In still another embodiment, the system 10 includes a living cell, e.g., cultured cells, e.g., primary cell or transformed cells. In yet another embodiment, the system includes a living organism. The organism includes a cell 15 that can express the target protein.

The method can further include contacting the test compound to an organism (e.g., *C. elegans*, *Drosophila*, or a mammal, e.g., a mouse) and evaluating an age-associated parameter of the organism.

Exemplary properties include a catalytic parameter; structural conformation; post-translational modification; redox state; physical interaction of the target protein with another protein; metabolite formation or consumption; subcellular localization of the target protein; *in vivo* half-life of the target protein or target protein activity; transcription of a gene 20 encoding the target protein or translation of the target protein.

In one embodiment, the catalytic parameter describes the catalytic properties of an enzyme, other than the target protein. For example, the target protein is a substrate of the enzyme. In one embodiment, the post-translational modification is a modification of the target protein. In another embodiment, the post-translational modification is catalyzed by the 25 target protein. Exemplary post-translational modifications include phosphorylation, ubiquitination, methylation, acetylation/deacetylation, geranygeranylation, farnesylation, or proteolytic modification. In one embodiment, where the property relates to metabolite production or consumption, the metabolite can be a direct substrate or direct product of a reaction catalyzed or effected by the target protein. In another embodiment, where the 30 property relates to metabolite production or consumption, the metabolite can be an indirect substrate or indirect product of a reaction catalyzed or effected by the target protein.

A culture cell used in the method can include a heterologous nucleic acid that encodes and expresses the target protein. The method can further include assessing whether the test compound directly interacts with the target protein.

In one embodiment, the target protein is operably linked to a reporter protein and the evaluating comprises evaluating the reporter protein.

In one embodiment, the target protein is a cytochrome, and the property is redox state of the cytochrome.

5 In another aspect, the invention features a method that includes providing a nematode in which activity of a target protein is reduced in the organism by RNA interference; expressing a gene encoding a protein (e.g., candidate protein) that is heterologous to the organism; and evaluating an age associated parameter of the organism. The method can further include, prior to the evaluating, contacting the organism with a test 10 compound. The candidate protein can be, for example, a mammalian protein. The candidate protein can be, for example, a respiratory chain component. The method can include other features described herein.

15 In another aspect, the invention features a method that includes: providing a cell in which activity of a target protein is reduced in the organism by RNA interference; expressing a gene encoding a protein (e.g., candidate protein) that is heterologous to the organism; and evaluating an age associated parameter of the organism. The method can include other features described herein.

20 In still another aspect, the invention features a method that includes providing a cell or an organism (e.g., a nematode) in which activity of a target protein is reduced in the cell or one or more cells of the organism by RNA interference; contacting the organism or the cell with a test compound; and evaluating an age associated parameter of the organism.

25 In another aspect, the invention features a method that includes assessing an age-related parameter of a nematode that (1) expresses a heterologous gene in at least some cells; and (2) is deficient in at least some cells for an endogenous activity provided by a respiratory chain component. In one embodiment, the heterologous gene is from a non-nematode species, e.g., a mammalian species. In a related embodiment, the heterologous gene encodes a variant of a mammalian protein, the variant having between one and ten substitutions, insertions, or deletions. The heterologous gene can encode a domain of at least 30 amino acids from a mammalian protein or a variant thereof, having between one and six 30 substitutions, insertions, or deletions.

In one embodiment, the endogenous activity is provided by a respiratory chain component (e.g., cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5)), a GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2

(SEQ ID NO:8), LLW-3 (SEQ ID NO:9); LLW-4 (SEQ ID NO:10); HSF-1 (SEQ ID NO:11), or a protein listed in Table 5 or 6.

The method can include contacting the organism with a test compound, e.g., prior to the assessing. The method can include other features described herein.

In another aspect, the invention features a method of characterizing a protein, the method includes: providing a nucleic acid that encodes a protein having a subject amino acid sequence that contains at least one substitution, insertion, or deletion relative to a reference amino acid sequence, wherein the subject amino acid sequence and the reference amino acid sequence are at least 70% identical; expressing the nucleic acid in a culture cell or in an invertebrate cell; and evaluating an age-associated parameter of the cell, or an organism that includes the cell. The reference amino acid sequence can be selected from a sequence described herein, e.g., one of the following sequences cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), LLW-4 (SEQ ID NO:10); HSF-1 (SEQ ID NO:11), the amino acid sequences of proteins listed in Table 5 and 6, and human homologues.

In another aspect, the invention features a *C. elegans* nematode that (1) expresses a heterologous gene in at least some cells, the heterologous gene encoding a heterologous protein (e.g., a protein described herein (e.g., a mammalian gene described herein) that is non-identical to a corresponding endogenous protein, or a functional domain thereof; and (2) is deficient in at least some cells for an endogenous activity provided by the corresponding endogenous protein. For example, the nematode can (1) express a heterologous gene in at least some cells, the heterologous gene encoding a respiratory chain component that is non-identical to the corresponding endogenous respiratory chain component, or a functional domain thereof; and (2) be deficient in at least some cells for an endogenous activity provided by the corresponding endogenous respiratory chain component.

In one embodiment, the heterologous gene encodes a mammalian protein. In another embodiment, the heterologous gene encodes a variant of a mammalian protein, the variant having between one and ten substitutions, insertions, or deletions. For example, the heterologous gene encodes at least a domain of at least 30 amino acids from a mammalian protein or a variant thereof, having between one and six substitutions, insertions, or deletions. The deficiency can be mediated by dsRNA, e.g., by RNA interference.

The invention also features a method that includes assessing an age-associated parameter of a nematode described herein, e.g., a nematode described above. In one embodiment, the method further includes, prior to the assessing, contacting the nematode to a test compound.

In another aspect, the invention features a *C. elegans* nematode that (1) is deficient in at least some cells for an endogenous activity, the deficiency generated by dsRNA in the cells, and (2) has an average lifespan of at least 24, 26, or 28 days (e.g., in the N2 background) or an average lifespan of at least 25% greater than the average lifespan of an otherwise identical nematode not contacted with the dsRNA. The nematode is such that, absent the deficiency, the nematode has an average lifespan of less than 22, 20, 18, or 16 days. In one embodiment, the nematode has functional genes for dauer pathway and/or functional genes for *clk-1*, *gro-1*, and/or another gene described herein. For example, the nematode can be wild-type with respect to a laboratory standard, e.g., the N2 background or another background. The dsRNA may include a strand that is complementary to a nucleic acid encoding a protein described herein, e.g., comprising an amino acid sequence selected from the group consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9); LLW-4 (SEQ ID NO:10); and HSF-1 (SEQ ID NO:11), proteins listed in Table 5 and 6, and human homologues corresponding to all of the above.

In another aspect, the invention features a method that includes: providing a nematode described herein, e.g., a nematode described above; introducing a heterologous gene that encodes a polypeptide into the nematode; expressing the heterologous gene in the nematode or a progeny of the nematode under conditions wherein the polypeptide is produced; and monitoring an age-associated parameter of the nematode or the progeny of the nematode. The heterologous gene can be, e.g., a nematode gene or a mammalian gene.

In another aspect, the invention features a method includes: providing a library of a test agents; contacting each test agent of the library to cells; evaluating expression of a HSF-regulated gene in the contacted cells; for each test agent that alters the expression of a HSF-regulated gene, contacting a test organism with the test compound; and evaluating an age-associated parameter of the test organism. Many genes regulated by HSF are known or can be readily identified. See, e.g., GuhaThakurta *et al.* *Genome Res* 12(5):701-12 (2002) and Christians *et al.* *Crit Care Med* 2002 Jan;30(1 Suppl):S43-50 (2002).

In another aspect, the invention features a nucleic acid vector that includes (1) a coding sequence encoding an amino acid sequence comprising SEQ ID NO:7, 8, 9, or 10 or a fragment thereof; and (2) one or more of the following: (a) a promoter that is operably linked and heterologous to the coding sequence, and (b) a second coding sequence encoding a reporter protein or protein tag, the second coding sequence being operably linked and heterologous to the coding sequence. The invention also features a dsRNA (e.g., an isolated dsRNA) that comprises a nucleic acid segment that is complementary to a *C. elegans* gene that encodes SEQ ID NO:7, 8, 9, or 10, wherein the dsRNA, when administered to a *C. elegans* or cell thereof, causes an extension of lifespan of at least 20%. In one embodiment, the dsRNA is not complementary to a homologue (e.g., a homologue described herein) of the *C. elegans* gene that encodes SEQ ID NO:7, 8, 9, or 10. The invention also provides a method that includes providing (e.g., feeding) an adult nematode a dsRNA described herein. The invention also features an antibody that binds to SEQ ID NO:7, 8, 9, or 10. In a related aspect, the invention features a nucleic acid vector that includes (1) a coding sequence encoding an amino acid sequence comprising to a gene listed in Table 5 or 6; and (2) one or more of the following: (a) a promoter that is operably linked and heterologous to the coding sequence, and (b) a second coding sequence encoding a reporter protein or protein tag, the second coding sequence being operably linked and heterologous to the coding sequence.

The invention also features a method that includes: providing a cell, organism, or biochemical system that includes a subject protein described herein (e.g., a protein comprising an amino acid sequence selected from the group consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9); LLW-4 (SEQ ID NO:10); and HSF-1 (SEQ ID NO:11), and proteins described in Tables 5 and 6, or human homologues thereof); contacting an antibody that binds to the subject protein or antigen-binding fragment thereof to the cell or the organism; and evaluating an age-associated parameter of the cell, organism, or biochemical system.

30 DEFINITIONS

As is known to those of skill in the art, "oxidative phosphorylation" or "mitochondrial respiratory chain protein" and "glycolysis" proteins are involved in metabolic energy pathways that are universal in eukaryotic biological systems. The terms "oxidative phosphorylation or mitochondrial respiratory chain protein," e.g., cytochrome C1 (SEQ ID

NO:1 Accession No. CAA99820.1); NADH oxidoreductase (SEQ ID NO:2 Accession No. AAB522474.1); ATP synthase (SEQ ID NO:3 Accession No. AAB37654.1); and cytochrome C oxidase (SEQ ID NO:4 Accession No. CAB03002.1); or a "glycolysis protein," e.g., phosphoglucose isomerase, hexose isomerase, or glucose phosphate isomerase (SEQ ID NO:5 Accession No. CAB60430.1); or "GTPase" e.g., (SEQ ID NO:6 , Accession No. AAC48200.1); or "heat shock factor," e.g. HSF-1 (SEQ ID NO:11, Accession No. CAA22146); or "Long-lived worm protein," e.g. LLW-1 (SEQ ID NO:7, Accession No. CAA22452), LLW-2 (SEQ ID NO:8, Accession No. AAB54251), LLW-3 (SEQ ID NO:9, Accession No. CAB07688), LLW-4 (SEQ ID NO:10, Accession No. CAB04386), or a nucleic acid encoding a "mitochondrial respiratory chain protein, e.g., cytochrome C1, cytochrome C oxidase, NADH oxidoreductase, and ATP synthase" or a "glycolysis protein, e.g., phosphoglucose isomerase," or a "GTPase" or a "heat shock factor" or a "long-lived worm protein, e.g., llw-1, llw-2, llw-3, llw-4" as well as the genes and gene products listed in Table 5 and Table 6 refer to eukaryotic and mammalian nucleic acids and polypeptide polymorphic variants, alleles, mutants, and interspecies homologs that: (1) have an amino acid sequence that has greater than about 50% amino acid sequence identity, preferably 60%, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or greater amino acid sequence identity, preferably over a region of at least about 25, 50, 100, 200, 500, 1000, or more amino acids, to an amino acid sequence encoded by the *C. elegans* genes provided herein; (2) bind to antibodies, e.g., polyclonal antibodies, raised against an immunogen comprising an amino acid sequence of a *C. elegans* mitochondrial respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein, or gene product listed in Table 5 or 6 provided herein, and conservatively modified variants thereof; (3) specifically hybridize under moderately stringent hybridization conditions to an anti-sense strand corresponding to a nucleic acid sequence encoding an *C. elegans* mitochondrial respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein or gene listed in Table 5 or Table 6 as provided herein, and conservatively modified variants thereof; (4) have a nucleic acid sequence that has greater than about 50%, preferably greater than about 60%, 65%, 70%, 75%, 80%, 85%, 90%, preferably 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% or higher nucleotide sequence identity, preferably over a region of at least about 25, 50, 100, 200, 500, 1000, or more nucleotides, to a *C. elegans* mitochondrial respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein as provided herein; and/or (5) sequences that genetically complement the *C. elegans* loss of function of a mitochondrial

respiratory chain, glycolysis protein, GTPase, heat shock factor, or long-lived worm protein or gene listed in Table 5 or Table 6, as provided herein. A polynucleotide or polypeptide sequence is typically from a eukaryote, e.g., an invertebrate, vertebrate, or plant, preferably a mammal including, but not limited to, primate, e.g., human; rodent, e.g., rat, mouse, hamster; 5 cow, pig, horse, sheep, or any mammal. The nucleic acids and proteins of the invention include both naturally occurring or recombinant molecules. The sequence of the *C. elegans* genome can be found in *Science* 282:2012-2018 (1998).

The definition explicitly includes the human or mammalian homologues or counterparts of each *C. elegans* aging associated gene or protein described herein, e.g., 10 cytochrome C1, gene C54G4.8 (SEQ ID NO:1, Accession No. CAA99820.1), NADH oxidoreductase, gene T10E9.7 (SEQ ID NO:2, Accession No. AAB522474.1), ATP synthase, gene F27C1.7 (SEQ ID NO:3, Accession No. AAB37654.1), cytochrome C oxidase, gene F26E4.9 (SEQ ID NO:4, Accession No. CAB03002.1), GTPase, gene T23H2.5; (SEQ ID NO:6, Accession No. AAC48200.1) *llw-1*, gene Y54G11A.8 (SEQ ID NO:7, Accession No. 15 CAA22452); *llw-2*, gene F59E12.10 (SEQ ID NO:8, Accession No. AAB54251); *llw-3*, gene Y48E1B.1 (SEQ ID NO:9, Accession No. CAB07688); *llw-4*, gene F45H10.4 (SEQ ID NO:10, Accession No. CAB04386); HSF-1, gene Y53C10A.12 (SEQ ID NO:11, Accession No. CAA22146), and the genes and gene products listed in Tables 5 and 6.

The phrase "functional effects" in the context of assays for testing compounds 20 that modulate activity of aging associated genes and proteins includes the determination of a parameter that is indirectly (e.g., upstream or downstream biochemical or genetic effects) or directly under the influence of aging associated proteins, e.g., a chemical or phenotypic effect, such as the ability to increase or decrease lifespan (*see, e.g.*, Kenyon *et al.*, *Nature* 366:461-464 (1993); Hsin & Kenyon, *Nature* 399:362-366 (1999); Apfeld & Kenyon, *Cell* 25 95:199-210 (1998); and Lin *et al.*, *Nature Genet.* 28:139-145 (2001)) or, e.g., a physical effect such as ligand, cofactor or substrate binding or inhibition of ligand, cofactor or substrate binding. A functional effect therefore includes ligand, cofactor and substrate binding activity; changes in gene expression and gene expression levels in cells; changes in post transcriptional modification of a protein, e.g., phosphorylation or glycosylation; reporter 30 gene or marker expression; changes in abundance and cellular localization; enzymatic activity; cellular half life; redox state; and structural conformation, etc.; and age-associated parameters, i.e., characteristics of young or old cells or organisms such as stress resistance, lifespan, doubling time, telomere length, physiological characteristics, appearance, disease states, etc. "Functional effects" include *in vitro*, *in vivo*, and *ex vivo* activities. The

functional effect can be measured in a host cell, organelle (e.g., isolated mitochondria), host cell membrane, isolated organelle membrane (e.g., isolated mitochondrial membrane), cellular extract, organelle extract (e.g., mitochondrial extract) or host organism.

By "determining the functional effect" is meant assaying for a compound that increases or decreases a parameter that is indirectly or directly under the influence of aging associated proteins or genes, e.g., measuring physical and chemical or phenotypic effects. Such functional effects can be measured by any means known to those skilled in the art, e.g., changes in spectroscopic characteristics (e.g., fluorescence, absorbance, refractive index); hydrodynamic (e.g., shape); chromatographic; or solubility properties for the protein; measuring inducible markers or transcriptional activation of the protein; measuring binding activity or binding assays, e.g. binding to antibodies; measuring changes in ligand binding activity; measuring cellular proliferation or lifespan; measuring cell surface marker expression; measurement of changes in protein levels for associated sequences; measurement of RNA stability; phosphorylation or dephosphorylation; signal transduction, e.g., receptor-ligand interactions, second messenger concentrations (e.g., cAMP, IP₃, or intracellular Ca²⁺); identification of downstream or reporter gene expression (CAT, luciferase, β-gal, GFP and the like), e.g., via chemiluminescence, fluorescence, colorimetric reactions, antibody binding, inducible markers, and ligand binding assays.

"Ligand" refers to a molecule that is specifically bound by a protein.

"Substrate" refers to a molecule that binds to an enzyme and is part of a specific chemical reaction catalyzed by the enzyme.

"Cofactor" refers to an additional component required for activity of an enzyme. (Leninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). A cofactor may be inorganic such as Fe, Cu, K, Ni, Mo, Se, Zn, Mn or Mg ions, or an organic molecule also known as a coenzyme. Coenzymes include flavin adenine dinucleotide (FAD), nicotinamide adenine dinucleotide (NAD), nicotinamide adenine dinucleotide phosphate (NADP), heme, coenzyme A, pyrodoxal phosphate, thiamine pyrophosphate, 5'-deoxyadenosylcobalamin, biocytin, tetrahydrofolate, retinal, and 1,25-dihydroxycholecalciferol. A co-factor can also include a protein subunit bound to the co-factor.

"Inhibitors", "activators", and "modulators" of aging associated genes and proteins are used to refer to activating, inhibitory, or modulating molecules identified using *in vitro* and *in vivo* assays of aging associated proteins and genes. Inhibitors are compounds that, e.g., bind to, partially or totally block activity, decrease, prevent, delay activation,

inactivate, desensitize, or down regulate the activity or expression of aging associated proteins and genes, e.g., antagonists. "Activators" are compounds that increase, open, activate, facilitate, enhance activation, sensitize, agonize, or up regulate aging associated proteins. Inhibitors, activators, or modulators also include genetically modified versions of aging associated proteins and genes, e.g., versions with altered activity, as well as naturally occurring and synthetic ligands, antagonists, agonists, antibodies, antisense molecules, ribozymes, small chemical molecules and the like. Such assays for inhibitors and activators include, e.g., expressing aging associated proteins *in vitro*, in cells, or cell membranes, applying putative modulator compounds, and then determining the functional effects on activity, as described above.

Samples or assays comprising aging associated proteins and genes that are treated with a potential activator, inhibitor, or modulator are compared to control samples without the inhibitor, activator, or modulator to examine the extent of inhibition. Control samples (untreated with inhibitors) are assigned a relative protein or gene activity value of 100%. Inhibition of aging associated proteins or genes is achieved when the activity value relative to the control is about 80%, preferably 50%, more preferably 25-0%. Activation of aging associated proteins or genes is achieved when the activity value relative to the control (untreated with activators) is 110%, more preferably 150%, more preferably 200-500% (i.e., two to five fold higher relative to the control), more preferably 1000-3000% higher.

The term "test compound" or "drug candidate" or "modulator" or grammatical equivalents as used herein describes any molecule, either naturally occurring or synthetic, e.g., protein, oligopeptide (e.g., from about 5 to about 25 amino acids in length, preferably from about 10 to 20 or 12 to 18 amino acids in length, preferably 12, 15, or 18 amino acids in length), small organic molecule, polysaccharide, lipid, fatty acid, polynucleotide, oligonucleotide, etc., to be tested for the capacity to directly or indirectly modulation lymphocyte activation. The test compound can be in the form of a library of test compounds, such as a combinatorial or randomized library that provides a sufficient range of diversity. Test compounds are optionally linked to a fusion partner, e.g., targeting compounds, rescue compounds, dimerization compounds, stabilizing compounds, addressable compounds, and other functional moieties. Conventionally, new chemical entities with useful properties are generated by identifying a test compound (called a "lead compound") with some desirable property or activity, e.g., inhibiting activity, creating variants of the lead compound, and evaluating the property and activity of those variant compounds. Often, high throughput screening (HTS) methods are employed for such an analysis.

A "small organic molecule" refers to an organic molecule, either naturally occurring or synthetic, that has a molecular weight of more than about 50 daltons and less than about 2500 daltons, preferably less than about 2000 daltons, preferably between about 100 to about 1000 daltons, more preferably between about 200 to about 500 daltons.

5 "Biological sample" include sections of tissues such as biopsy and autopsy samples, and frozen sections taken for histologic purposes. Such samples include blood, sputum, tissue, cultured cells, e.g., primary cultures, explants, and transformed cells, stool, urine, etc. A biological sample is typically obtained from a eukaryotic organism, e.g., *C. elegans*, most preferably a mammal such as a primate e.g., chimpanzee or human; cow; dog; cat; a rodent, e.g., guinea pig, rat, mouse; or a rabbit.

10 The terms "identical" or percent "identity," in the context of two or more nucleic acids or polypeptide sequences, refer to two or more sequences or subsequences that are the same or have a specified percentage of amino acid residues or nucleotides that are the same (i.e., about 50% identity, preferably 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 15 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region (e.g., the *C. elegans* proteins provided herein), when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection. Such sequences are then said to be 20 "substantially identical." This definition also refers to, or may be applied to, the compliment of a test sequence. The definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. As described below, the preferred algorithms can account for gaps and the like. Preferably, identity exists over a region that is at least about 25 amino acids or nucleotides in length, or more preferably over a region that is 25 50-100 amino acids or nucleotides in length.

For sequence comparison, typically one sequence acts as a reference sequence, to which test sequences are compared. When using a sequence comparison algorithm, test and reference sequences are entered into a computer, subsequence coordinates are designated, if necessary, and sequence algorithm program parameters are designated. Preferably, default 30 program parameters can be used, or alternative parameters can be designated. The sequence comparison algorithm then calculates the percent sequence identities for the test sequences relative to the reference sequence, based on the program parameters.

A "comparison window", as used herein, includes reference to a segment of any one of the number of contiguous positions selected from the group consisting of from 20

to 600, usually about 50 to about 200, more usually about 100 to about 150 in which a sequence may be compared to a reference sequence of the same number of contiguous positions after the two sequences are optimally aligned. Methods of alignment of sequences for comparison are well-known in the art. Optimal alignment of sequences for comparison can be conducted, e.g., by the local homology algorithm of Smith & Waterman, *Adv. Appl. Math.* 2:482 (1981), by the homology alignment algorithm of Needleman & Wunsch, *J. Mol. Biol.* 48:443 (1970), by the search for similarity method of Pearson & Lipman, *Proc. Nat'l. Acad. Sci. USA* 85:2444 (1988), by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Dr., Madison, WI), or by manual alignment and visual inspection (see, e.g., *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds. 1995 supplement)).

A preferred example of algorithm that is suitable for determining percent sequence identity and sequence similarity are the BLAST and BLAST 2.0 algorithms, which are described in Altschul *et al.*, *Nuc. Acids Res.* 25:3389-3402 (1977) and Altschul *et al.*, *J. Mol. Biol.* 215:403-410 (1990), respectively. BLAST and BLAST 2.0 are used, with the parameters described herein, to determine percent sequence identity for the nucleic acids and proteins of the invention. Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a word of the same length in a database sequence. T is referred to as the neighborhood word score threshold (Altschul *et al.*, *supra*). These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value; the cumulative score goes to zero or below, due to the accumulation of one or more negative-scoring residue alignments; or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation

(E) of 10, M=5, N=-4 and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength of 3, and expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff, *Proc. Natl. Acad. Sci. USA* 89:10915 (1989)) alignments (B) of 50, expectation (E) of 10, M=5, N=-4, and a comparison of both 5 strands.

“Nucleic acid” refers to deoxyribonucleotides or ribonucleotides and polymers thereof in either single- or double-stranded form. The term encompasses nucleic acids containing known nucleotide analogs or modified backbone residues or linkages, which are synthetic, naturally occurring, and non-naturally occurring, which have similar binding 10 properties as the reference nucleic acid, and which are metabolized in a manner similar to the reference nucleotides. Examples of such analogs include, without limitation, phosphorothioates, phosphoramidates, methyl phosphonates, chiral-methyl phosphonates, 2-O-methyl ribonucleotides, peptide-nucleic acids (PNAs).

Unless otherwise indicated, a particular nucleic acid sequence also implicitly 15 encompasses conservatively modified variants thereof (e.g., degenerate codon substitutions) and complementary sequences, as well as the sequence explicitly indicated. Specifically, degenerate codon substitutions may be achieved by generating sequences in which the third position of one or more selected (or all) codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res.* 19:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem.* 260:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* 8:91-98 (1994)). The 20 term nucleic acid is used interchangeably with gene, cDNA, mRNA, oligonucleotide, and polynucleotide.

A particular nucleic acid sequence also implicitly encompasses “splice 25 variants.” Similarly, a particular protein encoded by a nucleic acid implicitly encompasses any protein encoded by a splice variant of that nucleic acid. “Splice variants,” as the name suggests, are products of alternative splicing of a gene. After transcription, an initial nucleic acid transcript may be spliced such that different (alternate) nucleic acid splice products encode different polypeptides. Mechanisms for the production of splice variants vary, but include alternate splicing of exons. Alternate polypeptides derived from the same nucleic acid by read-through transcription are also encompassed by this definition. Any products of a splicing reaction, including recombinant forms of the splice products, are included in this 30 definition.

The terms “polypeptide,” “peptide” and “protein” are used interchangeably herein to refer to a polymer of amino acid residues. The terms apply to amino acid polymers

in which one or more amino acid residue is an artificial chemical mimetic of a corresponding naturally occurring amino acid, as well as to naturally occurring amino acid polymers and non-naturally occurring amino acid polymer.

The term "amino acid" refers to naturally occurring and synthetic amino acids, 5 as well as amino acid analogs and amino acid mimetics that function in a manner similar to the naturally occurring amino acids. Naturally occurring amino acids are those encoded by the genetic code, as well as those amino acids that are later modified, e.g., hydroxyproline, γ -carboxyglutamate, and O-phosphoserine. Amino acid analogs refers to compounds that have the same basic chemical structure as a naturally occurring amino acid, i.e., an α carbon that is 10 bound to a hydrogen, a carboxyl group, an amino group, and an R group, e.g., homoserine, norleucine, methionine sulfoxide, methionine methyl sulfonium. Such analogs have modified R groups (e.g., norleucine) or modified peptide backbones, but retain the same basic chemical structure as a naturally occurring amino acid. Amino acid mimetics refers to chemical 15 compounds that have a structure that is different from the general chemical structure of an amino acid, but that functions in a manner similar to a naturally occurring amino acid.

Amino acids may be referred to herein by either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUB Biochemical Nomenclature Commission. Nucleotides, likewise, may be referred to by their commonly accepted single-letter codes.

"Conservatively modified variants" applies to both amino acid and nucleic acid sequences. With respect to particular nucleic acid sequences, conservatively modified variants refers to those nucleic acids which encode identical or essentially identical amino acid sequences, or where the nucleic acid does not encode an amino acid sequence, to 20 essentially identical sequences. Because of the degeneracy of the genetic code, a large number of functionally identical nucleic acids encode any given protein. For instance, the 25 codons GCA, GCC, GCG and GCU all encode the amino acid alanine. Thus, at every position where an alanine is specified by a codon, the codon can be altered to any of the corresponding codons described without altering the encoded polypeptide. Such nucleic acid variations are "silent variations," which are one species of conservatively modified 30 variations. Every nucleic acid sequence herein which encodes a polypeptide also describes every possible silent variation of the nucleic acid. One of skill will recognize that each codon in a nucleic acid (except AUG, which is ordinarily the only codon for methionine, and TGG, which is ordinarily the only codon for tryptophan) can be modified to yield a functionally identical molecule. Accordingly, each silent variation of a nucleic acid which encodes a

polypeptide is implicit in each described sequence with respect to the expression product, but not with respect to actual probe sequences.

As to amino acid sequences, one of skill will recognize that individual substitutions, deletions or additions to a nucleic acid, peptide, polypeptide, or protein sequence which alters, adds or deletes a single amino acid or a small percentage of amino acids in the encoded sequence is a "conservatively modified variant" where the alteration results in the substitution of an amino acid with a chemically similar amino acid. Conservative substitution tables providing functionally similar amino acids are well known in the art. Such conservatively modified variants are in addition to and do not exclude polymorphic variants, interspecies homologs, and alleles of the invention.

The following eight groups each contain amino acids that are conservative substitutions for one another: 1) Alanine (A), Glycine (G); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (K); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W); 7) Serine (S), Threonine (T); and 8) Cysteine (C), Methionine (M) (see, e.g., Creighton, *Proteins* (1984)).

Macromolecular structures such as polypeptide structures can be described in terms of various levels of organization. For a general discussion of this organization, see, e.g., Alberts *et al.*, *Molecular Biology of the Cell* (3rd ed., 1994) and Cantor and Schimmel, *Biophysical Chemistry Part I: The Conformation of Biological Macromolecules* (1980). "Primary structure" refers to the amino acid sequence of a particular peptide. "Secondary structure" refers to locally ordered, three dimensional structures within a polypeptide. These structures are commonly known as domains, e.g., extracellular domains, transmembrane domains, and cytoplasmic domains. Domains are portions of a polypeptide that form a compact unit of the polypeptide and are typically 15 to 350 amino acids long. Typical domains are made up of sections of lesser organization such as stretches of β -sheet and α -helices. "Tertiary structure" refers to the complete three dimensional structure of a polypeptide monomer. "Quaternary structure" refers to the three dimensional structure formed by the noncovalent association of independent tertiary units. Anisotropic terms are also known as energy terms.

A "label" or a "detectable moiety" is a composition detectable by spectroscopic, photochemical, biochemical, immunochemical, chemical, or other physical means. For example, useful labels include ^{32}P , fluorescent dyes, electron-dense reagents, enzymes (e.g., as commonly used in an ELISA), biotin, digoxigenin, or haptens and proteins

which can be made detectable, e.g., by incorporating a radiolabel into the peptide or used to detect antibodies specifically reactive with the peptide.

The term "recombinant" when used with reference, e.g., to a cell, or nucleic acid, protein, or vector, indicates that the cell, nucleic acid, protein or vector, has been modified by the introduction of a heterologous nucleic acid or protein or the alteration of a native nucleic acid or protein, or that the cell is derived from a cell so modified. Thus, for example, recombinant cells express genes that are not found within the native (non-recombinant) form of the cell or express native genes that are otherwise abnormally expressed, under expressed or not expressed at all.

The term "heterologous" when used with reference to portions of a nucleic acid indicates that the nucleic acid comprises two or more subsequences that are not found in the same relationship to each other in nature. For instance, the nucleic acid is typically recombinantly produced, having two or more sequences from unrelated genes arranged to make a new functional nucleic acid, e.g., a promoter from one source and a coding region from another source. Similarly, a heterologous protein indicates that the protein comprises two or more subsequences that are not found in the same relationship to each other in nature (e.g., a fusion protein).

The phrase "stringent hybridization conditions" refers to conditions under which a probe will hybridize to its target subsequence, typically in a complex mixture of nucleic acids, but to no other sequences. Stringent conditions are sequence-dependent and will be different in different circumstances. Longer sequences hybridize specifically at higher temperatures. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Probes*, "Overview of principles of hybridization and the strategy of nucleic acid assays" (1993). Generally, stringent conditions are selected to be about 5-10°C lower than the thermal melting point (T_m) for the specific sequence at a defined ionic strength pH. The T_m is the temperature (under defined ionic strength, pH, and nucleic concentration) at which 50% of the probes complementary to the target hybridize to the target sequence at equilibrium (as the target sequences are present in excess, at T_m , 50% of the probes are occupied at equilibrium). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. For selective or specific hybridization, a positive signal is at least two times background, preferably 10 times background hybridization.

Exemplary stringent hybridization conditions can be as following: 50% formamide, 5x SSC, and 1% SDS, incubating at 42°C, or, 5x SSC, 1% SDS, incubating at

65°C, with wash in 0.2x SSC, and 0.1% SDS at 65°C. For PCR, a temperature of about 36°C is typical for low stringency amplification, although annealing temperatures may vary between about 32°C and 48°C depending on primer length. For high stringency PCR amplification, a temperature of about 62°C is typical, although high stringency annealing temperatures can range from about 50°C to about 65°C, depending on the primer length and specificity. Typical cycle conditions for both high and low stringency amplifications include a denaturation phase of 90°C - 95°C for 30 sec - 2 min., an annealing phase lasting 30 sec. - 2 min., and an extension phase of about 72°C for 1 - 2 min. Protocols and guidelines for low and high stringency amplification reactions are provided, e.g., in Innis *et al.* (1990) *PCR Protocols, A Guide to Methods and Applications*, Academic Press, Inc. N.Y.).

Nucleic acids that do not hybridize to each other under stringent conditions are still substantially identical if the polypeptides which they encode are substantially identical. This occurs, for example, when a copy of a nucleic acid is created using the maximum codon degeneracy permitted by the genetic code. In such cases, the nucleic acids typically hybridize under moderately stringent hybridization conditions. Exemplary "moderately stringent hybridization conditions" include a hybridization in a buffer of 40% formamide, 1 M NaCl, 1% SDS at 37°C, and a wash in 1X SSC at 45°C. A positive hybridization is at least twice background. Those of ordinary skill will readily recognize that alternative hybridization and wash conditions can be utilized to provide conditions of similar stringency. Additional guidelines for determining hybridization parameters are provided in numerous reference, e.g., and Current Protocols in Molecular Biology, ed. Ausubel, *et al*

"Antibody" refers to a polypeptide comprising a framework region from an immunoglobulin gene or fragments thereof that specifically binds and recognizes an antigen. The recognized immunoglobulin genes include the kappa, lambda, alpha, gamma, delta, epsilon, and mu constant region genes, as well as the myriad immunoglobulin variable region genes. Light chains are classified as either kappa or lambda. Heavy chains are classified as gamma, mu, alpha, delta, or epsilon, which in turn define the immunoglobulin classes, IgG, IgM, IgA, IgD and IgE, respectively. Typically, the antigen-binding region of an antibody will be most critical in specificity and affinity of binding.

An exemplary immunoglobulin (antibody) structural unit comprises a tetramer. Each tetramer is composed of two identical pairs of polypeptide chains, each pair having one "light" (about 25 kD) and one "heavy" chain (about 50-70 kD). The N-terminus of each chain defines a variable region of about 100 to 110 or more amino acids primarily

responsible for antigen recognition. The terms variable light chain (V_L) and variable heavy chain (V_H) refer to these light and heavy chains respectively.

Antibodies exist, e.g., as intact immunoglobulins or as a number of well-characterized fragments produced by digestion with various peptidases. Thus, for example, 5 pepsin digests an antibody below the disulfide linkages in the hinge region to produce $F(ab')_2$, a dimer of Fab which itself is a light chain joined to V_H-C_H1 by a disulfide bond. The $F(ab')_2$ may be reduced under mild conditions to break the disulfide linkage in the hinge region, thereby converting the $F(ab')_2$ dimer into an Fab' monomer. The Fab' monomer is 10 essentially Fab with part of the hinge region (see *Fundamental Immunology* (Paul ed., 3d ed. 1993). While various antibody fragments are defined in terms of the digestion of an intact antibody, one of skill will appreciate that such fragments may be synthesized *de novo* either chemically or by using recombinant DNA methodology. Thus, the term antibody, as used herein, also includes antibody fragments either produced by the modification of whole 15 antibodies, or those synthesized *de novo* using recombinant DNA methodologies (e.g., single chain Fv) or those identified using phage display libraries (see, e.g., McCafferty et al., *Nature* 348:552-554 (1990))

For preparation of antibodies, e.g., recombinant, monoclonal, or polyclonal antibodies, many technique known in the art can be used (see, e.g., Kohler & Milstein, *Nature* 256:495-497 (1975); Kozbor et al., *Immunology Today* 4: 72 (1983); Cole et al., pp. 20 77-96 in *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss, Inc. (1985); Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *Antibodies, A Laboratory Manual* (1988); and Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986)). Techniques for the production of single chain antibodies (U.S. Patent 4,946,778) can be adapted to produce antibodies to polypeptides of this invention. Also, transgenic mice, or 25 other organisms such as other mammals, may be used to express humanized antibodies. Alternatively, phage display technology can be used to identify antibodies and heteromeric Fab fragments that specifically bind to selected antigens (see, e.g., McCafferty et al., *Nature* 348:552-554 (1990); Marks et al., *Biotechnology* 10:779-783 (1992)).

A "chimeric antibody" is an antibody molecule in which (a) the constant 30 region, or a portion thereof, is altered, replaced or exchanged so that the antigen binding site (variable region) is linked to a constant region of a different or altered class, effector function and/or species, or an entirely different molecule which confers new properties to the chimeric antibody, e.g., an enzyme, toxin, hormone, growth factor, drug, etc.; or (b) the variable

region, or a portion thereof, is altered, replaced or exchanged with a variable region having a different or altered antigen specificity.

In one embodiment, the antibody is conjugated to an "effector" moiety. The effector moiety can be any number of molecules, including labeling moieties such as radioactive labels or fluorescent labels, or can be a therapeutic moiety. In one aspect the antibody modulates the activity of the protein.

The phrase "specifically (or selectively) binds" to an antibody or "specifically (or selectively) immunoreactive with," when referring to a protein or peptide, refers to a binding reaction that is determinative of the presence of the protein, often in a heterogeneous population of proteins and other biologics. Thus, under designated immunoassay conditions, the specified antibodies bind to a particular protein at least two times the background and more typically more than 10 to 100 times background. Specific binding to an antibody under such conditions requires an antibody that is selected for its specificity for a particular protein. For example, polyclonal antibodies raised to aging associated proteins, polymorphic variants, alleles, orthologs, and conservatively modified variants, or splice variants, or portions thereof, can be selected to obtain only those polyclonal antibodies that are specifically immunoreactive with aging associated proteins and not with other proteins. This selection may be achieved by subtracting out antibodies that cross-react with other molecules. A variety of immunoassay formats may be used to select antibodies specifically immunoreactive with a particular protein. For example, solid-phase ELISA immunoassays are routinely used to select antibodies specifically immunoreactive with a protein (see, e.g., Harlow & Lane, *Antibodies, A Laboratory Manual* (1988) for a description of immunoassay formats and conditions that can be used to determine specific immunoreactivity).

25 ASSAYS FOR GENES AND GENE PRODUCTS THAT REGULATE AGING

Genetic and other models can be used to identify mutants, phenotypes (mediated by mutants and by RNAi), genes, and gene products in the aging process, e.g., RNAi analysis; microarray analysis; chemical mutagenesis; mammalian complementation assays for age-associated proteins; yeast two hybrid assays, immunoprecipitation; alteration in age-associated reporter gene expression or localization (e.g., daf-2 or daf-16); overexpression, underexpression, or knockout of gene expression, etc. Suitable controls include organisms with altered lifespan, e.g., by mutation or RNAi. These assays can be used with eukaryotic organisms, cells, and organelles such as mitochondria. The genes and gene products associated with a mutation are then identified and used to analyze the aging process

at a molecular level. Genes and gene products that regulate the aging process can be identified under normal aging conditions. Patterns of gene expression that correlate with normal or abnormal aging can also be used to identify genes associated with aging. The aging process has likely been conserved throughout evolution. Thus, genes and gene products that regulate the aging process in one species will be useful to identify similar or orthologous genes and gene products in divergent species.

A. *Manifestations of the Aging Process*

The most obvious disruption of the aging process is a change in lifespan of an individual. Lifespan can either be increased or decreased by a mutation in a gene that participates in the aging process or, as shown here, by another intervention, e.g., RNAi mediated silencing of such a gene. In addition, for all eukaryotic organisms other physical characteristics can be used to distinguish young individuals from older individuals. Thus, at an organismal level, a mutation that affects the aging process will usually affect the lifespan of an individual and may also affect other aging characteristics of that individual. Such manifestations of the aging process are known as "age-associated parameters," e.g., indicia from Nomarski analysis, stress resistance, appearance, physiological changes, disease states, loss of doubling capacity, changes in differentiated phenotype, indirect effects such as fusion protein expression and localization or posttranscriptional modification, etc., are described in more detail below.

Those of skill in the art will recognize that the aging process can also be manifested at an organismal level or at a cellular level. While a list of characteristics of aging is provided below, it is not exhaustive and other characteristics of the aging process may also be analyzed within the scope of the present invention.

Characteristics of aging can be distinguished at the organismal level and may be species specific. For example, characteristics of older human individuals include skin wrinkling, graying of the hair, baldness, cataracts, hypermelanosis, osteoporosis, cerebral cortical atrophy, lymphoid depletion, thymic atrophy, increased incidence of diabetes type II, atherosclerosis, cancer, and heart disease (Nehlin *et al.*, *Annals NY Acad. Sci.*, 980:176-179 (2000)). Other characteristics of mammalian aging include the following: weight loss; lordokyphosis (hunchback spine); absence of vigor; lymphoid atrophy; decreases in bone density, dermal thickness, and subcutaneous adipose tissue; decreased ability to tolerate stresses, such as wound healing, anesthesia, and response to hematopoietic precursor cell

ablation; sparse hair; liver pathologies; atrophy of intestinal villi; skin ulceration; amyloid deposits; and joint diseases (Tyner *et al.*, *Nature* 415:45-53 (2002)).

Careful observation reveals characteristics of the aging process in other eukaryotes, including invertebrates. For example, characteristics of aging in the model 5 nematode *C. elegans* as observed by Nomarski analysis include slow movement, flaccidity, yolk accumulation, intestinal autofluorescence (lipofuscin), loss of ability to chew and expel (distended oral and anal cavities), necrotic cavities in tissue, curdled appearing tissue, and germ cell appearance (graininess, large, well separated nuclei, fewer nuclei, and cavities).

Characteristics of aging can also be observed in cultured cells and also in 10 mitochondria. Note that many of these characteristics can also be observed in animals. Normal eukaryotic cells have a defined lifespan when taken from the organism grown in culture. These "primary" tissue culture cells are cells that have neither been immortalized nor acquired a transformed phenotype. The primary cells will divide a defined number of times in culture and then die (reviewed in Campisi, *Exper. Geron.* 36:6-7-618 (2001)). 15 Cellular aging is also characterized by changes other than loss of doubling capability, e.g. changes in apoptotic death and changes in differentiated phenotypes (*Id.*). In some cases, cellular characteristics of aging can also be observed in immortalized or transformed cell lines. Aging cells also show stress resistance, e.g., free radical generation and H₂O₂ resistance. Age-related bio-markers, gene, and protein expression patterns may also be used 20 to determine or measure aging.

Finally, aging can be assessed indirectly, by an aging related functional effects 25 (phenotypic, physical, and chemical effects), e.g., gene expression (e.g., transcript abundance), protein abundance/localization/modification state, chromatin structure, signal transduction, second messenger levels, marker expression, phosphorylation, posttranscriptional modification, reporter gene expression, reporter or fusion protein localization, etc. Such effects can often be monitored when examining upstream or downstream genetic or biochemical pathways of an aging associated gene. Such effects can also be monitored using the aging associated gene.

In one embodiment, a test compound is contacted to one or more cells of an 30 organism or one or more culture cells, and the one or more cells, or the entire organism is evaluated. In particular, a characteristic of aging (e.g., a direct observation or an aging-related functional effect) can be evaluated to determine the test compound has an affect on aging or an aging-related process such as stress resistance or metabolism.

B. *Isolation of Genes Associated with Aging*

Those of skill in the art will recognize that aging associated nucleic acids and proteins may be conserved in divergent species. Thus, the sequence of a nucleic acid or protein associated with aging in one species can be used to identify aging associated nucleic acids and proteins from other species, as well as genetic and biochemical pathways for the aging associated genes. For example, using methods described in this specification, aging associated genes identified in *C. elegans* can be used to identify aging associated genes or proteins in humans or other higher eukaryotes.

10 Isolation of genes and gene products associated with aging using classical genetic methods.

Using classical genetic methods (random genomic mutagenesis), aging mutants are generated by mutagenesis. The mutagenesis protocol will depend on the organism. For example, some eukaryotic organisms can be randomly mutagenized chemically by treatment with compounds like ethane methyl sulfonate (EMS) or can be mutagenized by exposure to UV or gamma irradiation. Preferably, these compounds would be used on organisms such as mammalian cells, yeast, *C. elegans*, *Drosophila melanogaster*, or zebrafish.

20 Mutants in the aging process will preferably be characterized by an increase or a decrease in lifespan. Mutants in the aging process will also preferably exhibit a temporal change in expression of an aging characteristic, including those listed above. Those of skill in the art will recognize that mutants can be generated in many ways depending on the organism and phenotypes studied. Typically, the mutagenesis process decreases, increases, or changes gene activity. Examples of such mutants include age-1, daf-2, and daf-16 in *C. elegans*.

25 Isolation of genes and gene products associated with aging using gene inactivation.

In another embodiment, aging mutants are made by inactivation of a gene of interest, using methods other than classical genetic mutagenesis methods. The gene of interest can be inactivated, e.g., using dsRNA inhibition, by using antisense technology, or can be inactivated by homologous recombination. The inactivation can take place in a multicellular organism or in cultured cells. For example, the p66 gene has been removed from mice using homologous recombination, creating a mouse with a longer lifespan than

wildtype. Transgenic mice of interest which show lifespan increase include Ames dwarf mutant mice, p66(-/-) knockout mice, alpha MUPA and MGMT transgenic mice (see, e.g., Anisimov, *Mech Aging Dev.* 122:1221-1255 (2001); Lithgow & Andersen, *Bioessays* 22:410-413 (2000)).

5 dsRNA inhibition can also be used to screen a large number of genes for a phenotype. The method is preferably done in an organism whose genome has been sequenced. DNA fragments corresponding to predicted genes are cloned into a vector between two bacterial promoters in inverted orientation. The library is then transformed into a bacterial strain capable of expressing the DNA fragments. The transformed bacteria or the 10 library DNA alone is then introduced into the experimental organism. If desired, inducible promoters can be used and expression of the inhibitory dsRNA can be induced during a particular time of development or under desired conditions.

15 A preferred embodiment uses a library whose members each include a DNA fragment from *C. elegans*. Each library member is transformed into *E. coli* and the *E. coli* fed to the worms. The DNA fragments are under the control of T7 promoters. The bacteria express a T7 polymerase that is inducible by IPTG, rendering expression of the inhibitory dsRNA inducible by IPTG.

20 Isolation of genes and gene products associated with aging using overexpression.

In another embodiment, aging mutants are made by overexpressing a gene associated with aging, using methods other than classical genetic mutagenesis methods. The gene associated with aging is cloned into a vector under the control of a promoter appropriate for the experimental system. The expression vector is then introduced into the experimental 25 system. The overexpression can take place in either a multicellular organism or in cultured cells.

30 Isolation of genes and gene products associated with aging using naturally occurring mutants.

Aging mutants can also occur naturally. Those of skill in the art will recognize that such mutants do exist and can be used in the present invention. For example, in humans, several premature aging syndromes have been characterized including Werner syndrome, Hutchinson-Guilford disease, Bloom's syndrome, Cockayne's syndrome, ataxia

telangiectasia, and Down's syndrome. Where appropriate, cells from an individual afflicted with an aging syndrome can be studied, rather than the whole organism.

5 Isolation of genes and gene products associated with aging using genetic or biochemical pathways known to regulate aging.

Genetic analysis can also be used to delineate regulatory pathways and determine functional relationships between genes and gene products. In the case of a complex biological process such as aging, more than one regulatory pathway may regulate the aging process. Those of skill in the art will recognize that genetic analysis of mutants can 10 be used to characterize regulatory pathways and determine relationships between genes. Of course, it is also possible to use RNA interference to modulate gene activity in analyzing the regulatory pathways and relationships.

An example of genetic analysis of a regulatory pathway is found in *C. elegans*. The *daf-2* gene encodes an insulin/IGF-1 receptor homologue. Mutations that lower the level 15 of *daf-2* result in animals that have enhanced lifespans. (For review see Guarente and Kenyon, *Nature* 408:255-262 (2000)). *daf-16* encodes a forkhead transcription factor homologue that acts downstream of *daf-2* and is required for *daf-2* activity. *daf-16* mutants 20 have short lifespans. Newly isolated mutations can be analyzed for interaction with the *daf2/daf16* pathway. In that way, genes and gene products can be assigned to a regulatory pathway.

In addition, genes that interact with the pathway can be identified by using an appropriate mutant screen. For example, the *C. elegans* protein DAF-16 is a transcriptional activator. A fusion protein between DAF-16 and green fluorescent protein (DAF-16/GFP) 25 can be used to identify the cellular location of the protein. In wild-type animals the protein is localized throughout cells. In long-lived *daf-2* mutants, DAF-16 is localized to the nucleus.

Those of skill in the art will recognize that the localization of DAF-16/GFP can be used to identify mutants that perturb the *daf2/daf16* pathway. Localization of DAF-16/GFP to the nucleus can be used to screen for drugs that enhance lifespan or mutations that enhance lifespan.

30 A similar fusion using an end product of the pathway, superoxide dismutase (SOD-3), was constructed. Long lived mutants of the *daf-2/daf-16* pathway expressed SOD-3/GFP at a higher level than wild-type worms. Levels of fluorescence from SOD-3/GFP can be followed by microscopy. Those of skill in the art will recognize that expression of SOD-3/GFP can be used to screen for long-lived mutants.

Isolation of genes and gene products associated with aging using changes in expression levels.

Those of skill in the art will recognize that levels of messenger RNA can be measured during the aging process. For aging associated proteins, changes in mRNA levels can be detected either during normal aging process or when comparing an aging mutant to a wild-type individual. Changes in mRNA levels can be measured using techniques known to those of skill in the art, including microarrays, northern blots, and RT PCR.

Aging associated genes can be identified through the use of microarrays where changes in expression of mRNA levels under different conditions or at different times of development can be assayed (see Tables 5 and 6). mRNA levels can also be analyzed in aging mutants to identify genes that are affected by increases or decreases in lifespan.

Microarrays are made by methods known to those of skill in the art, or are purchased. Gene expression profiles for the genes described herein can be generated and used for comparison to identify other age-associated genes. The profile can be generated using a microarray, or by other means. The profiles can be derived from animals, cells, mitochondria, or other suitable sources expressing the genes of interest, e.g., RNAi treated cells or animals. Such profiles can be stored as computer files and analyzed or compared to identify additional genes using algorithms known to those of skill in the art.

Moreover, a gene identified by any method, e.g., transcript or protein profiling, RNAi, or genetic mutation, can then be analyzed by one of the other methods. For example, the activity of a gene whose transcription is correlated with aging can be altered using RNAi. Further, chromosomal deficiencies and genetic mutations can be identified in the gene of interest. These exemplary alterations can be used to evaluate the contribution of a gene or gene product to the aging phenotype. The functional relevance of genes so identified can be tested with mutants or RNAi.

COMPUTER ASSISTED DRUG DESIGN AND PROFILING

Yet another assay for compounds that modulate aging involves computer assisted drug design, in which a computer system is used to generate a three-dimensional structure of an aging associated protein based on the structural information encoded by the amino acid or nucleic acid sequence. The input amino acid sequence interacts directly and actively with a pre-established algorithm in a computer program to yield secondary, tertiary, and quaternary structural models of the protein. The models of the protein structure are then

examined to identify regions of the structure that have the ability to bind, e.g., ligands, substrates, cofactors, etc. These regions are then used to identify ligands that bind to the protein.

The three-dimensional structural model of the protein is generated by entering 5 an aging associated protein amino acid sequences of at least 25, 50, 75 or 100 amino acid residues or corresponding nucleic acid sequences encoding an aging associated protein into the computer system. The amino acid sequence represents the primary sequence or subsequence of each of the proteins, which encodes the structural information of the protein. At least 25, 50, 75, or 100 residues of the amino acid sequence (or a nucleotide sequence 10 encoding at least about 25, 50, 75 or 100 amino acids) are entered into the computer system from computer keyboards, computer readable substrates that include, but are not limited to, electronic storage media (e.g., magnetic diskettes, tapes, cartridges, and chips), optical media (e.g., CD ROM), information distributed by internet sites, and by RAM. The three-dimensional structural model of the aging-associated protein is then generated by the 15 interaction of the amino acid sequence and the computer system, using software known to those of skill in the art. The resulting three-dimensional computer model can then be saved on a computer readable substrate. For example, three-dimensional models of the structures of a number of proteins described here are known and can be used to model homologs and interactions with other chemical compounds. See, e.g., Damberger *et al. Protein Sci.* 1994 20 Oct;3(10):1806-21. (HSF structure); Harrison *et al. Science.* 1994 Jan 14;263(5144):224-7 (HSF structure); Lange *et al. Proc Natl Acad Sci U S A.* 2002 Mar 5;99(5):2800-5 (cytochrome bc₁ complex); Iwata, *et al., Science.* 1998 Jul 3;281(5373):64-71; Gibbons *et al., Nat Struct Biol.* 2000 Nov;7(11):1055-61 (F(1)-ATPase); Faig *et al., (2001) Structure* (Camb). 2001 Aug;9(8):659-67 (NAD(P)H:quinone oxidoreductase 1); Ingelman *et al. Biochemistry.* 1999 Jun 1;38(22):7040-9. (NAD(P)H:flavin oxidoreductase).

The amino acid sequence represents a primary structure that encodes the 30 information necessary to form the secondary, tertiary and quaternary structure of the aging associated protein. The software looks at certain parameters encoded by the primary sequence to generate the structural model. These parameters are referred to as "energy terms," or anisotropic terms and primarily include electrostatic potentials, hydrophobic potentials, solvent accessible surfaces, and hydrogen bonding. Secondary energy terms include van der Waals potentials. Biological molecules form the structures that minimize the energy terms in a cumulative fashion. The computer program is therefore using these terms

encoded by the primary structure or amino acid sequence to create the secondary structural model.

The tertiary structure of the protein encoded by the secondary structure is then formed on the basis of the energy terms of the secondary structure. The user at this point can enter additional variables such as whether the protein is membrane bound or soluble, its location in the body, and its cellular location, e.g., cytoplasmic, surface, or nuclear. These variables along with the energy terms of the secondary structure are used to form the model of the tertiary structure. In modeling the tertiary structure, the computer program matches hydrophobic faces of secondary structure with like, and hydrophilic faces of secondary structure with like.

Once the structure has been generated, potential ligand and substrate binding regions are identified by the computer system. Three-dimensional structures for potential ligands are generated by entering amino acid or nucleotide sequences or chemical formulas of compounds, as described above. The three-dimensional structure of the potential ligand is then compared to that of the aging associated protein to identify ligands that bind to the aging associated protein, orthologs thereof, etc. Binding affinity between the protein and ligands is determined using energy terms to determine which ligands have an enhanced probability of binding to the protein.

Computer systems are also used to screen for mutations, polymorphic variants, alleles and interspecies homologs of the aging associated protein or gene. Such mutations can be associated with disease states. Once the variants are identified, diagnostic assays can be used to identify patients having such mutated genes associated with disease states. Identification of the mutated aging associated protein involves receiving input of a first nucleic acid, e.g., SEQ ID NOS:1-11, and genes disclosed in Tables 5 and 6 and orthologs or conservatively modified versions thereof. The sequence is entered into the computer system as described above. The first nucleic acid or amino acid sequence is then compared to a second nucleic acid or amino acid sequence that has substantial identity to the first sequence. The second sequence is entered into the computer system in the manner described above. Once the first and second sequences are compared, nucleotide or amino acid differences between the sequences are identified. Such sequences can represent allelic differences in aging associated protein, e.g., human genes and mutations associated with disease states. The first and second sequences described above can be saved on a computer readable substrate.

Nucleic acids encoding aging associated proteins can be used with high density oligonucleotide array technology (e.g., GeneChipTM) to identify family members and

homologs, orthologs, alleles, conservatively modified variants, and polymorphic variants in this invention. In the case where the homologs being identified are linked to a known disease, they can be used with GeneChip™ as a diagnostic tool in detecting the disease in a biological sample, *see, e.g.*, Gunthand *et al.*, *AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al.*, *Nat. Med.* 2:753-759 (1996); Matson *et al.*, *Anal. Biochem.* 224:110-106 (1995); Lockhart *et al.*, *Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al.*, *Genome Res.* 8:435-448 (1998); Hacia *et al.*, *Nucleic Acids Res.* 26:3865-3866 (1998).

In another aspect, the invention features a computer medium having a plurality of digitally encoded data records. Each data record includes a value representing the level of expression of one or more age-associated gene as described herein in a sample, and a descriptor of the sample. The level of expression can relate to mRNA level and/or protein levels. The record can further include an aging-associated parameter as described herein. The descriptor of the sample can indicate a subject from which the sample was derived (e.g., a patient, a mutant animal), a treatment (e.g., RNAi treatment), or a location of the sample. In one embodiment, the data record further includes values representing the level of expression of genes and proteins other than an age-associated gene of the invention (e.g., other genes associated with an aging-disorder, or other genes on an array). The data record can be structured as a table, e.g., a table that is part of a database such as a relational database (e.g., a SQL database of the Oracle or Sybase database environments).

Also featured is a method of evaluating a sample. The method includes providing a sample, e.g., from the subject, and determining an expression profile of the sample, wherein the profile includes a value representing the level expression of an age-associated gene described herein. The method can further include comparing the value or the profile (i.e., multiple values) to a reference value or reference profile. The gene expression profile of the sample can be obtained by any of the methods described herein (e.g., by providing a nucleic acid from the sample and contacting the nucleic acid to an array). The method can be used to infer a longevity-associated phenotype in a subject wherein an increase or decrease expression of an age-associated gene described herein is an indication that the subject has or is disposed to having an altered longevity-associated phenotype. The method can be used to monitor a treatment for an aging in a subject. For example, the gene expression profile can be determined for a sample from a subject undergoing treatment. The profile can be compared to a reference profile or to a profile obtained from the subject prior to treatment or prior to onset of the disorder (*see, e.g.*, Golub *et al.*, *Science* 286:531 (1999)).

ISOLATION OF NUCLEIC ACIDS ENCODING AGING ASSOCIATED PROTEINS

This invention can include use of routine techniques in the field of recombinant genetics. Basic texts disclosing the general methods of use in this invention include Sambrook *et al.*, *Molecular Cloning, A Laboratory Manual* (2nd ed. 1989); Kriegler, *Gene Transfer and Expression: A Laboratory Manual* (1990); and *Current Protocols in Molecular Biology* (Ausubel *et al.*, eds., 1994)).

Aging associated protein-encoding nucleic acids, polymorphic variants, orthologs, and alleles can be isolated using the *C. elegans* genes provided herein using, e.g., moderate or low stringent hybridization conditions, by screening libraries, by analyzing a sequence database, and/or by synthetic gene construction. Alternatively, expression libraries can be used to clone aging associated proteins, polymorphic variants, orthologs, and alleles by detecting expressed homologs immunologically with antisera or purified antibodies made against *C. elegans* or mammalian aging associated proteins or portions thereof or by complementation, e.g., of a *C. elegans* phenotype. In a preferred embodiment, human nucleic acid libraries are screened for homologues of *C. elegans* genes or proteins that are associated with aging.

To make a cDNA library, one can choose a source that is rich in the RNA of choice. The mRNA is then made into cDNA using reverse transcriptase, ligated into a recombinant vector, and transfixed into a recombinant host for propagation, screening and cloning. Methods for making and screening cDNA libraries are well known (see, e.g., Gubler & Hoffman, *Gene* 25:263-269 (1983); Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

For a genomic library, the DNA is extracted from the tissue and either mechanically sheared or enzymatically digested to yield fragments of about 12-20 kb. The fragments are then separated by gradient centrifugation from undesired sizes and are constructed in bacteriophage lambda vectors. These vectors and phage are packaged *in vitro*. Recombinant phage are analyzed by plaque hybridization as described in Benton & Davis, *Science* 196:180-182 (1977). Colony hybridization is carried out as generally described in Grunstein *et al.*, *Proc. Natl. Acad. Sci. USA.*, 72:3961-3965 (1975).

An alternative method of isolating aging associated protein-encoding nucleic acid and their orthologs, alleles, mutants, polymorphic variants, and conservatively modified variants combines the use of synthetic oligonucleotide primers and amplification of an RNA or DNA template (see U.S. Patents 4,683,195 and 4,683,202; *PCR Protocols: A Guide to Methods and Applications* (Innis *et al.*, eds, 1990)). Methods such as polymerase chain reaction (PCR) and ligase chain reaction (LCR) can be used to amplify nucleic acid

sequences of aging associated protein-encoding genes directly from mRNA, from cDNA, from genomic libraries or cDNA libraries. Degenerate oligonucleotides can be designed to amplify homologs using the sequences provided herein. Restriction endonuclease sites can be incorporated into the primers. Polymerase chain reaction or other *in vitro* amplification methods may also be useful, for example, to clone nucleic acid sequences that code for proteins to be expressed, to make nucleic acids to use as probes for detecting the presence of aging associated protein encoding mRNA in physiological samples, for nucleic acid sequencing, or for other purposes. Genes amplified by the PCR reaction can be purified from agarose gels and cloned into an appropriate vector.

Gene expression of aging associated proteins can also be analyzed by techniques known in the art, e.g., reverse transcription and amplification of mRNA, isolation of total RNA or poly A⁺ RNA, northern blotting, dot blotting, *in situ* hybridization, RNase protection, high density polynucleotide array technology, e.g., and the like.

Nucleic acids encoding aging associated proteins can be used with high density oligonucleotide array technology (e.g., GeneChipTM) to identify aging associated proteins, orthologs, alleles, conservatively modified variants, and polymorphic variants in this invention. In the case where the homologs being identified are linked to modulation of aging associated proteins, they can be used with GeneChipTM as a diagnostic tool in detecting the disease in a biological sample, *see, e.g.*, Gunthand *et al.*, *AIDS Res. Hum. Retroviruses* 14: 869-876 (1998); Kozal *et al.*, *Nat. Med.* 2:753-759 (1996); Matson *et al.*, *Anal. Biochem.* 224:110-106 (1995); Lockhart *et al.*, *Nat. Biotechnol.* 14:1675-1680 (1996); Gingeras *et al.*, *Genome Res.* 8:435-448 (1998); Hacia *et al.*, *Nucleic Acids Res.* 26:3865-3866 (1998).

The gene for aging associated proteins are typically cloned into intermediate vectors before transformation into prokaryotic or eukaryotic cells for replication and/or expression. These intermediate vectors are typically prokaryote vectors, e.g., plasmids, or shuttle vectors.

EXPRESSION IN PROKARYOTES AND EUKARYOTES

To obtain high level expression of a cloned gene, such as those cDNAs encoding aging associated proteins, one typically subclones aging associated protein encoding nucleic acids into an expression vector that contains a strong promoter to direct transcription, a transcription/translation terminator, and a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and described, *e.g.*, in Sambrook *et al.*, and Ausubel *et al.*, *supra*. Bacterial expression systems for

expressing aging associated proteins are available in, e.g., *E. coli*, *Bacillus sp.*, and *Salmonella* (Palva et al., *Gene* 22:229-235 (1983); Mosbach et al., *Nature* 302:543-545 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available.

Selection of the promoter used to direct expression of a heterologous nucleic acid depends on the particular application. The promoter is preferably positioned about the same distance from the heterologous transcription start site as it is from the transcription start site in its natural setting. As is known in the art, however, some variation in this distance can be accommodated without loss of promoter function.

In addition to the promoter, the expression vector typically contains a transcription unit or expression cassette that contains all the additional elements required for the expression of aging associated protein encoding nucleic acid in host cells. A typical expression cassette thus contains a promoter operably linked to the nucleic acid sequence encoding aging associated proteins and signals required for efficient polyadenylation of the transcript, ribosome binding sites, and translation termination. Additional elements of the cassette may include enhancers and, if genomic DNA is used as the structural gene, introns with functional splice donor and acceptor sites.

In addition to a promoter sequence, the expression cassette should also contain a transcription termination region downstream of the structural gene to provide for efficient termination. The termination region may be obtained from the same gene as the promoter sequence or may be obtained from different genes.

The particular expression vector used to transport the genetic information into the cell is not particularly critical. Any of the conventional vectors used for expression in eukaryotic or prokaryotic cells may be used. Standard bacterial expression vectors include plasmids such as pBR322 based plasmids, pSKF, pET23D, and fusion expression systems such as MBP, GST, and LacZ. Epitope tags can also be added to recombinant proteins to provide convenient methods of isolation, e.g., c-myc.

Expression vectors containing regulatory elements from eukaryotic viruses are typically used in eukaryotic expression vectors, e.g., SV40 vectors, papilloma virus vectors, and vectors derived from Epstein-Barr virus. Other exemplary eukaryotic vectors include pMSG, pAV009/A⁺, pMTO10/A⁺, pMAMneo-5, baculovirus pDSVE, and any other vector allowing expression of proteins under the direction of the CMV promoter, SV40 early promoter, SV40 later promoter, metallothionein promoter, murine mammary tumor virus

promoter, Rous sarcoma virus promoter, polyhedrin promoter, or other promoters shown effective for expression in eukaryotic cells.

Expression of proteins from eukaryotic vectors can be also be regulated using inducible promoters. With inducible promoters, expression levels are tied to the concentration of inducing agents, such as tetracycline or ecdysone, by the incorporation of response elements for these agents into the promoter. Generally, high level expression is obtained from inducible promoters only in the presence of the inducing agent; basal expression levels are minimal. Inducible expression vectors are often chosen if expression of the protein of interest is detrimental to eukaryotic cells.

Some expression systems have markers that provide gene amplification such as thymidine kinase and dihydrofolate reductase. Alternatively, high yield expression systems not involving gene amplification are also suitable, such as using a baculovirus vector in insect cells, with mitochondrial respiratory chain protein encoding sequences and glycolysis protein encoding sequence under the direction of the polyhedrin promoter or other strong baculovirus promoters.

The elements that are typically included in expression vectors also include a replicon that functions in *E. coli*, a gene encoding antibiotic resistance to permit selection of bacteria that harbor recombinant plasmids, and unique restriction sites in nonessential regions of the plasmid to allow insertion of eukaryotic sequences. The particular antibiotic resistance gene chosen is not critical, any of the many resistance genes known in the art are suitable. The prokaryotic sequences are preferably chosen such that they do not interfere with the replication of the DNA in eukaryotic cells, if necessary.

Standard transfection methods are used to produce bacterial, mammalian, yeast or insect cell lines that express large quantities of aging associated proteins, which are then purified using standard techniques (see, e.g., Colley *et al.*, *J. Biol. Chem.* 264:17619-17622 (1989); *Guide to Protein Purification*, in *Methods in Enzymology*, vol. 182 (Deutscher, ed., 1990)). Transformation of eukaryotic and prokaryotic cells are performed according to standard techniques (see, e.g., Morrison, *J. Bact.* 132:349-351 (1977); Clark-Curtiss & Curtiss, *Methods in Enzymology* 101:347-362 (Wu *et al.*, eds, 1983)).

Any of the well-known procedures for introducing foreign nucleotide sequences into host cells may be used. These include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, biolistics, liposomes, microinjection, plasma vectors, viral vectors and any of the other well known methods for introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic material

into a host cell (*see, e.g.,* Sambrook *et al., supra*). It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing at least one gene into the host cell capable of expressing aging associated proteins.

After the expression vector is introduced into the cells, the transfected cells are cultured under conditions favoring expression of aging associated proteins, which is recovered from the culture using standard techniques identified below.

Expression vectors with appropriate regulatory sequences can also be used to express a heterologous gene in a nematode. In one example, the expression vector is injected in the gonad of the nematode, and the vector is incorporated, *e.g.*, as an extra-chromosomal array in progeny of the nematode. The vector can further include a second gene (*e.g.*, a marker gene) that indicates the presence of the vector. For example, the heterologous gene can be a mammalian gene, *e.g.*, a mammalian cDNA, or a fragment thereof.

PURIFICATION OF AGING ASSOCIATED PROTEINS

Either naturally occurring or recombinant aging associated proteins can be purified for use in functional assays. Naturally occurring aging associated proteins can be purified, *e.g.*, from human tissue. Recombinant aging associated proteins can be purified from any suitable expression system.

Aging associated proteins may be purified to substantial purity by standard techniques, including selective precipitation with such substances as ammonium sulfate; column chromatography, immunopurification methods, and others (*see, e.g.,* Scopes, *Protein Purification: Principles and Practice* (1982); U.S. Patent No. 4,673,641; Ausubel *et al., supra*; and Sambrook *et al., supra*).

A number of procedures can be employed when recombinant aging associated proteins are being purified. For example, proteins having established molecular adhesion properties can be reversible fused to aging associated proteins. With the appropriate ligand, aging associated proteins can be selectively adsorbed to a purification column and then freed from the column in a relatively pure form. The fused protein is then removed by enzymatic activity. Finally, aging associated proteins could be purified using immunoaffinity columns.

A. Purification of aging associated proteins from recombinant bacteria
Recombinant proteins are expressed by transformed bacteria in large amounts, typically after promoter induction; but expression can be constitutive. Promoter induction

with IPTG is one example of an inducible promoter system. Bacteria are grown according to standard procedures in the art. Fresh or frozen bacteria cells are used for isolation of protein.

Proteins expressed in bacteria may form insoluble aggregates ("inclusion bodies"). Several protocols are suitable for purification of aging associated protein inclusion bodies. For example, purification of inclusion bodies typically involves the extraction, separation and/or purification of inclusion bodies by disruption of bacterial cells, e.g., by incubation in a buffer of 50 mM TRIS/HCL pH 7.5, 50 mM NaCl, 5 mM MgCl₂, 1 mM DTT, 0.1 mM ATP, and 1 mM PMSF. The cell suspension can be lysed using 2-3 passages through a French Press, homogenized using a Polytron (Brinkman Instruments) or sonicated on ice. Alternate methods of lysing bacteria are apparent to those of skill in the art (see, e.g., 10 Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*).

If necessary, the inclusion bodies are solubilized, and the lysed cell suspension is typically centrifuged to remove unwanted insoluble matter. Proteins that formed the inclusion bodies may be renatured by dilution or dialysis with a compatible buffer. Suitable 15 solvents include, but are not limited to urea (from about 4 M to about 8 M), formamide (at least about 80%, volume/volume basis), and guanidine hydrochloride (from about 4 M to about 8 M). Some solvents which are capable of solubilizing aggregate-forming proteins, for example SDS (sodium dodecyl sulfate), 70% formic acid, are inappropriate for use in this procedure due to the possibility of irreversible denaturation of the proteins, accompanied by a 20 lack of immunogenicity and/or activity. Although guanidine hydrochloride and similar agents are denaturants, this denaturation is not irreversible and renaturation may occur upon removal (by dialysis, for example) or dilution of the denaturant, allowing re-formation of immunologically and/or biologically active protein. Other suitable buffers are known to those skilled in the art. Aging associated proteins are separated from other bacterial proteins 25 by standard separation techniques, e.g., with Ni-NTA agarose resin.

Alternatively, it is possible to purify aging associated proteins from bacteria periplasm. After lysis of the bacteria, when the aging associated proteins are exported into the periplasm of the bacteria, the periplasmic fraction of the bacteria can be isolated by cold osmotic shock in addition to other methods known to skill in the art. To isolate recombinant 30 proteins from the periplasm, the bacterial cells are centrifuged to form a pellet. The pellet is resuspended in a buffer containing 20% sucrose. To lyse the cells, the bacteria are centrifuged and the pellet is resuspended in ice-cold 5 mM MgSO₄ and kept in an ice bath for approximately 10 minutes. The cell suspension is centrifuged and the supernatant decanted.

and saved. The recombinant proteins present in the supernatant can be separated from the host proteins by standard separation techniques well known to those of skill in the art.

5 *B. Standard protein separation techniques for purifying aging associated proteins*

Solubility fractionation

Often as an initial step, particularly if the protein mixture is complex, an initial salt fractionation can separate many of the unwanted host cell proteins (or proteins derived from the cell culture media) from the recombinant protein of interest. The preferred salt is ammonium sulfate. Ammonium sulfate precipitates proteins by effectively reducing the amount of water in the protein mixture. Proteins then precipitate on the basis of their solubility. The more hydrophobic a protein is, the more likely it is to precipitate at lower ammonium sulfate concentrations. A typical protocol includes adding saturated ammonium sulfate to a protein solution so that the resultant ammonium sulfate concentration is between 10 20-30%. This concentration will precipitate the most hydrophobic of proteins. The precipitate is then discarded (unless the protein of interest is hydrophobic) and ammonium sulfate is added to the supernatant to a concentration known to precipitate the protein of interest. The precipitate is then solubilized in buffer and the excess salt removed if necessary, either through dialysis or diafiltration. Other methods that rely on solubility of 15 proteins, such as cold ethanol precipitation, are well known to those of skill in the art and can be used to fractionate complex protein mixtures.

20

Size differential filtration

The molecular weight of the aging associated proteins can be used to isolate it 25 from proteins of greater and lesser size using ultrafiltration through membranes of different pore size (for example, Amicon or Millipore membranes). As a first step, the protein mixture is ultrafiltered through a membrane with a pore size that has a lower molecular weight cut-off than the molecular weight of the protein of interest. The retentate of the ultrafiltration is then ultrafiltered against a membrane with a molecular cut off greater than the molecular weight of 30 the protein of interest. The recombinant protein will pass through the membrane into the filtrate. The filtrate can then be chromatographed as described below.

Column chromatography

The aging associated proteins can also be separated from other proteins on the basis of its size, net surface charge, hydrophobicity, and affinity for ligands. In addition, antibodies raised against proteins can be conjugated to column matrices and the proteins immunopurified. All of these methods are well known in the art. It will be apparent to one of skill that chromatographic techniques can be performed at any scale and using equipment from many different manufacturers (e.g., Pharmacia Biotech).

IMMUNOLOGICAL DETECTION OF AGING ASSOCIATED PROTEINS

In addition to the detection of aging associated genes and gene expression using nucleic acid hybridization technology, one can also use immunoassays to detect aging associated proteins of the invention. Such assays are useful for screening for modulators of aging associated proteins, e.g., for regulation of lifespan, as well as for therapeutic and diagnostic applications. Immunoassays can be used to qualitatively or quantitatively analyze aging associated proteins. A general overview of the applicable technology can be found in Harlow & Lane, *Antibodies: A Laboratory Manual* (1988).

Methods of producing polyclonal and monoclonal antibodies that react specifically with the aging associated proteins are known to those of skill in the art (see, e.g., Coligan, *Current Protocols in Immunology* (1991); Harlow & Lane, *supra*; Goding, *Monoclonal Antibodies: Principles and Practice* (2d ed. 1986); and Kohler & Milstein, *Nature* 256:495-497 (1975). Such techniques include antibody preparation by selection of antibodies from libraries of recombinant antibodies in phage or similar vectors, as well as preparation of polyclonal and monoclonal antibodies by immunizing rabbits or mice (see, e.g., Huse *et al.*, *Science* 246:1275-1281 (1989); Ward *et al.*, *Nature* 341:544-546 (1989)).

A number of immunogens comprising portions of aging associated proteins may be used to produce antibodies specifically reactive with an aging associated protein. For example, recombinant protein or an antigenic fragment thereof, can be isolated as described herein. Recombinant protein can be expressed in eukaryotic or prokaryotic cells as described above, and purified as generally described above. Recombinant protein is the preferred immunogen for the production of monoclonal or polyclonal antibodies. Alternatively, a synthetic peptide derived from the sequences disclosed herein and conjugated to a carrier protein can be used an immunogen. Naturally occurring protein may also be used either in pure or impure form. The product is then injected into an animal capable of producing

antibodies. Either monoclonal or polyclonal antibodies may be generated, for subsequent use in immunoassays to measure the protein.

Methods of production of polyclonal antibodies are known to those of skill in the art. An inbred strain of mice (e.g., BALB/C mice) or rabbits is immunized with the protein using a standard adjuvant, such as Freund's adjuvant, and a standard immunization protocol. The animal's immune response to the immunogen preparation is monitored by taking test bleeds and determining the titer of reactivity to the beta subunits. When appropriately high titers of antibody to the immunogen are obtained, blood is collected from the animal and antisera are prepared. Further fractionation of the antisera to enrich for antibodies reactive to the protein can be done if desired (see, Harlow & Lane, *supra*).

Monoclonal antibodies may be obtained by various techniques familiar to those skilled in the art. Briefly, spleen cells from an animal immunized with a desired antigen are immortalized, commonly by fusion with a myeloma cell (see, Kohler & Milstein, *Eur. J. Immunol.* 6:511-519 (1976)). Alternative methods of immortalization include transformation with Epstein Barr Virus, oncogenes, or retroviruses, or other methods well known in the art. Colonies arising from single immortalized cells are screened for production of antibodies of the desired specificity and affinity for the antigen, and yield of the monoclonal antibodies produced by such cells may be enhanced by various techniques, including injection into the peritoneal cavity of a vertebrate host. Alternatively, one may isolate DNA sequences which encode a monoclonal antibody or a binding fragment thereof by screening a DNA library from human B cells according to the general protocol outlined by Huse, *et al.*, *Science* 246:1275-1281 (1989).

Monoclonal antibodies and polyclonal sera are collected and titered against the immunogen protein in an immunoassay, for example, a solid phase immunoassay with the immunogen immobilized on a solid support. Typically, polyclonal antisera with a titer of 10^4 or greater are selected and tested for their cross reactivity against non-specific proteins, using a competitive binding immunoassay. Specific polyclonal antisera and monoclonal antibodies will usually bind with a K_d of at least about 0.1 mM, more usually at least about 1 μM , preferably at least about 0.1 μM or better, and most preferably, 0.01 μM or better. Antibodies specific only for a particular ortholog, such as a human ortholog, can also be made, by subtracting out other cross-reacting orthologs from a species such as a non-human mammal.

Once the specific antibodies against aging associated proteins are available, the protein can be detected by a variety of immunoassay methods. In addition, the antibody can be used therapeutically as aging associated protein modulators, e.g., to enhance and

extend lifespan or to prevent premature aging. For a review of immunological and immunoassay procedures, see *Basic and Clinical Immunology* (Stites & Terr eds., 7th ed. 1991). Moreover, the immunoassays of the present invention can be performed in any of several configurations, which are reviewed extensively in Enzyme Immunoassay (Maggio, ed., 1980); and Harlow & Lane, *supra*.

It is also possible to use protein arrays to detect an aging associated protein, e.g., to concurrently detect a plurality of aging associated proteins. Exemplary methods for producing protein arrays are provided in De Wildt *et al.* (2000) *Nat. Biotechnol.* 18:989-994; Lueking *et al.* (1999) *Anal. Biochem.* 270:103-111; Ge (2000) *Nucleic Acids Res.* 28, e3, I-VII; MacBeath and Schreiber (2000) *Science* 289:1760-1763; WO 0/98534, WO01/83827, WO02/12893, WO 00/63701, WO 01/40803 and WO 99/51773. In some implementations, polypeptides (including peptides) are spotted onto discrete addresses of the array, e.g., at high speed, e.g., using commercially available robotic apparatus, e.g., from Genetic MicroSystems or BioRobotics. The array substrate can be, for example, nitrocellulose, plastic, glass, e.g., surface-modified glass. The array can also include a porous matrix, e.g., acrylamide, agarose, or another polymer.

ASSAYS FOR MODULATORS OF AGING ASSOCIATED PROTEINS

A. Assays

Modulation of aging associated proteins and genes can be assessed using a variety of *in vitro* and *in vivo* assays, as described herein, and, such assays can be used to test for inhibitors and activators of aging associated proteins. Such modulators of aging associated proteins and genes, which are involved in aging, are useful for enhancing lifespan or treating premature aging. Modulators of aging associated proteins and genes are tested using either recombinant or naturally occurring, preferably *C. elegans*, mouse, rat, guinea pig, monkey, or human aging associated proteins.

An example of a modulator of the mitochondrial respiratory chain is antimycin. Antimycin inhibits the transfer of electrons from complex 2 to complex 3 during mitochondrial respiration. This drug has been found to extend the lifespan of *C. elegans*. Administration of antimycin to adult worms increased lifespan, while administration of antimycin to worms from hatching through adulthood substantially increases lifespan. Mean lifespan of untreated worms was 17 days while animals grown in the presence of 0.1 micromolar antimycin in ethanol had a mean lifespan of 21 days.

Preferably, the aging associated proteins or genes will have a *C. elegans* or a mammalian, e.g., a rat, mouse, guinea pig, rabbit, monkey, or human sequence. Alternatively, the aging associated proteins or genes of the assay will be derived from a eukaryote and include an nucleic acid or amino acid subsequence having sequence identity to the *C. elegans* genes and gene products described herein. Generally, the sequence identity will be at least 30%, 35%, 40%, 45% or 50%, preferably at least 55%, 60%, 65%, 70%, 75%, 80%, 85%, or 90%, most preferably at least 95%.

Measurement of modulation of aging phenotype with aging associated proteins or cells expressing aging associated proteins or genes, either recombinant or naturally occurring, can be performed using a variety of assays, *in vitro*, *in vivo*, and *ex vivo*. A suitable physiological change that affects activity can be used to assess the influence of a test compound on the polypeptide or nucleic acid of this invention. When the functional effects are determined using intact cells or animals, one can also measure a variety of effects such as, increases or decreases in lifespan, cellular proliferation, or in the case of signal transduction, hormone release, transcriptional changes to both known and uncharacterized genetic markers (e.g., northern blots), changes in cell metabolism such as cell growth or pH changes, and changes in intracellular second messengers such as cGMP.

In a preferred embodiment, aging associated protein or gene modulators are assayed *in vivo* by screening in *C. elegans* or in a mammalian model system (cellular or animal) for changes in mean and median lifespan.

Some aging associated proteins have measurable enzymatic activity. Thus, enzymatic assays can be performed to identify compounds that modulate the enzymatic activity. Enzymatic activity can encompass a chemical reaction carried out by a protein, as well as binding of substrates, cofactors, regulatory compounds, or ligands to the protein. It may also be useful to monitor the affect of a test compound on other properties of the aging associated protein, e.g., a structural property (e.g., conformation, oligomerization state, stability, mobility, and the like) or a cellular property (e.g., cellular localization, accessibility, clustering, and the like).

The protein activity and binding capabilities assayed will depend on the aging associated protein. For example, the following proteins with known enzymatic activities have been associated with aging in *C. elegans*: cytochrome C₁ (gene C54G4.8, SEQ ID NO:1 Accession No. CAA99820.1), NADH oxidoreductase (gene T10E9.7, SEQ ID NO:2 Accession No. AAB522474.1), ATP synthase (gene F27C1.7, SEQ ID NO:3 Accession No. AAB37654.1), cytochrome C oxidase (gene F26E4.9, SEQ ID NO:4 Accession No.

CAB03002.1), phosphoglucose isomerase (gene Y87G2A.8, SEQ ID NO:5 Accession No. CAB60430.1), a GTPase (gene T23H2.5, SEQ ID NO:6 Accession No. AAC48200.1), and HSF-1 (gene Y53C10A.12, SEQ ID NO:11 Accession No. CAA22146), and the genes and gene products listed in Table 5 and Table 6

5 For activity of cytochrome C and C1, binding to the cofactor heme is important. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). Thus, changes in ability to bind heme may correlate with a mutant phenotype or change during aging. Cytochrome C1 is an electron-transferring protein that contains heme and is found in cytochrome reductase, a membrane-associated proton pump. Cytochrome C is a 10 water soluble protein that transfers electrons from cytochrome reductase to cytochrome oxidase. Changes in electron transport may also be seen during aging or as a result of an aging mutation. It is possible to monitor the redox state of the heme cofactor, for example, by spectroscopy.

15 NADH oxidoreductase is an oligomeric enzyme complex located in the inner mitochondrial membrane. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). In *C. elegans* inhibition of the 30 Kd subunit resulted in enhanced lifespan. Thus, assays of NADH oxidoreductase activity could be associated with aging. It is possible to monitor the redox state of NADH, for example, by spectroscopy.

20 Cytochrome C oxidase catalyzes the transfer of electrons from cytochrome C to molecular oxygen. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). Those of skill in the art will recognize that this activity can be assayed spectrophotometrically. In addition to this activity, changes in binding to substrates cytochrome C and oxygen may also be assayed.

25 ATP synthase catalyzes the synthesis of ATP from ADP and orthophosphate. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). The energy of a proton gradient is used to release the ATP product from its binding site. The ATP synthase protein is a multisubunit enzyme. Inhibition of the *C. elegans* homologue of the delta subunit of ATP synthase resulted in an enhanced lifespan phenotype. Activity of the delta subunit is sensitive to the drug oligomycin. Stryer, page 546.

30 Phosphoglucose isomerase catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate. (Lehninger, *Principles of Biochemistry* (1984); Stryer, *Biochemistry* (1995)). The enzyme is involved in glycolysis in most higher organisms and in gluconeogenesis in mammals. An extracellular role has been reported for the protein as a nerve growth factor and cytokine. (Jeffery *et al.*, *Biochem.* 39:955-964 (2000)).

A GTPase enzyme catalyzes the hydrolysis of GTP to GDP. The reaction can be followed using radioactively labeled GTP and separating the reaction products from the reaction substrates.

HSF, a transcription factor, activates transcription by forming a tri-mer, 5 binding to a specific DNA sequence called a promoter and then recruiting RNA polymerases to begin transcription. Stress response and heat shock response proteins are known as chaperonins. Activation of transcription and binding to a target DNA site can be measured *in vitro* using purified or partially purified components. Activation of transcription can also be measured *in vivo* using a reporter gene construct with these proteins.

10 The functional activities described above do not represent all of the enzymatic activities that could be found in aging associated proteins. For example, some aging proteins could act to down regulate transcription of messenger RNA. Still other aging proteins may functional, e.g., as a structural scaffold or adaptor protein, e.g., they may or may not have an enzymatic activity.

15 Assays to identify compounds with modulating activity can be performed *in vitro*, e.g., in a test tube, or using isolated membranes, e.g., mitochondrial membranes, or using cellular or mitochondrial extracts. Exemplary assays can include, for example, methods described or referenced in Al-Awqati, *Annu. Rev. Cell Biol.* 2:179-199 (1986); Brand et al., *Biol. Rev. Cambridge Philosphic Soc.* 62:141-193 (1987); Capaldi et al., *FEBS Lett* 138:1-7 (1982); Casey, *Biochim. Biophys. Acta* 768:319-347 (1984); Erecinska et al., *J. Membr. Biol.* 70:1-14 (1982); Fillingame, *Annu. Rev. Biochem.* 49:1079-1113 (1980); Hamamoto, *Proc. Natl. Acad. Sci. USA* 82:2570-2573 (1985); Hatefi, *Annu. Rev. Biochem.* 54:1015-1070 (1985); Klingenberg, *Trends Biochem. Sci.* 4:249-252 (1979); LaNoue et al., *Annu. Rev. Biochem.* 48:871-922 (1979); Mitchell, *Nature* 191:144-148 (1961); Prince, *Trends Biochem. Sci.* 13:159-160 (1988); Slater, *Trends Biochem. Sci.* 8:239-242 (1983); Srere, *Trends Biochem. Sci.* 7:375-378 (1982); Tzagoloff, *Mitochondria*, New York: Plenum (1982); Weiss et al., *Biochem. Soc. Trans.* 15:100-102 (1987).

20 For example, the aging associated protein or gene is first contacted with a potential modulator and incubated for a suitable amount of time, e.g., from 0.5 to 48 hours. In one embodiment, aging associated protein or gene expression levels are determined *in vitro* by measuring the level of protein or mRNA. The level of protein or nucleic acid is measured using immunoassays such as western blotting, ELISA and the like with an antibody that selectively binds to the polypeptide or a fragment thereof. For measurement of mRNA, amplification, e.g., using PCR, LCR, or hybridization assays, e.g., northern hybridization,

RNAse protection, dot blotting, are preferred. The level of protein or mRNA is detected using directly or indirectly labeled detection agents, e.g., fluorescently or radioactively labeled nucleic acids, radioactively or enzymatically labeled antibodies, and the like, as described herein.

5 Alternatively, a reporter gene system can be devised using an aging associated protein promoter operably linked to a reporter gene such as chloramphenicol acetyltransferase, firefly luciferase, bacterial luciferase, β -galactosidase and alkaline phosphatase. Furthermore, the gene or protein of interest can be used as an indirect reporter via attachment to a second reporter such as green fluorescent protein (*see, e.g.*, Mistili & 10 Spector, *Nature Biotechnology* 15:961-964 (1997)). The reporter construct is typically transfected into a cell. After treatment with a potential modulator, the amount of reporter gene transcription, translation, or activity is measured according to standard techniques known to those of skill in the art.

15 B. *Modulators*

The compounds tested as modulators of aging associated proteins and genes can be any small chemical compound, or a biological entity, such as a protein, e.g., an antibody, a sugar, a nucleic acid, e.g., an antisense oligonucleotide or a ribozyme, or a lipid. Alternatively, modulators can be genetically altered versions of an aging associated proteins 20 and genes. Typically, test compounds will be small chemical molecules and peptides, or antibodies, antisense molecules, or ribozymes. Essentially any chemical compound can be used as a potential modulator or ligand in the assays of the invention, although most often compounds can be dissolved in aqueous or organic (especially DMSO-based) solutions are used. The assays are designed to screen large chemical libraries by automating the assay 25 steps and providing compounds from any convenient source to assays, which are typically run in parallel (e.g., in microtiter formats on microtiter plates in robotic assays). It will be appreciated that there are many suppliers of chemical compounds, including Sigma (St. Louis, MO), Aldrich (St. Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-Biochemica Analytika (Buchs Switzerland) and the like.

30 In one preferred embodiment, high throughput screening methods involve providing a combinatorial chemical or peptide library containing a large number of potential therapeutic compounds (potential modulator or ligand compounds). Such "combinatorial chemical libraries" or "ligand libraries" are then screened in one or more assays, as described

herein, to identify those library members (particular chemical species or subclasses) that display a desired characteristic activity. The compounds thus identified can serve as conventional "lead compounds" or can themselves be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of diverse chemical 5 compounds generated by either chemical synthesis or biological synthesis, by combining a number of chemical "building blocks" such as reagents. For example, a linear combinatorial chemical library such as a polypeptide library is formed by combining a set of chemical building blocks (amino acids) in every possible way for a given compound length (i.e., the 10 number of amino acids in a polypeptide compound). Millions of chemical compounds can be synthesized through such combinatorial mixing of chemical building blocks.

Preparation and screening of combinatorial chemical libraries is well known to those of skill in the art. Such combinatorial chemical libraries include, but are not limited to, peptide libraries (see, e.g., U.S. Patent 5,010,175, Furka, *Int. J. Pept. Prot. Res.* 37:487-493 15 (1991) and Houghton *et al.*, *Nature* 354:84-88 (1991)). Other chemistries for generating chemical diversity libraries can also be used. Such chemistries include, but are not limited to: 20 peptoids (e.g., PCT Publication No. WO 91/19735), encoded peptides (e.g., PCT Publication No. WO 93/20242), random bio-oligomers (e.g., PCT Publication No. WO 92/00091), benzodiazepines (e.g., U.S. Pat. No. 5,288,514), diversomers such as hydantoins, benzodiazepines and dipeptides (Hobbs *et al.*, *Proc. Nat. Acad. Sci. USA* 90:6909-6913 25 (1993)), vinylogous polypeptides (Hagihara *et al.*, *J. Amer. Chem. Soc.* 114:6568 (1992)), nonpeptidal peptidomimetics with glucose scaffolding (Hirschmann *et al.*, *J. Amer. Chem. Soc.* 114:9217-9218 (1992)), analogous organic syntheses of small compound libraries (Chen *et al.*, *J. Amer. Chem. Soc.* 116:2661 (1994)), oligocarbamates (Cho *et al.*, *Science* 261:1303 30 (1993)), and/or peptidyl phosphonates (Campbell *et al.*, *J. Org. Chem.* 59:658 (1994)), nucleic acid libraries (see Ausubel, Berger and Sambrook, all *supra*), peptide nucleic acid libraries (see, e.g., U.S. Patent 5,539,083), antibody libraries (see, e.g., Vaughn *et al.*, *Nature Biotechnology*, 14(3):309-314 (1996) and PCT/US96/10287), carbohydrate libraries (see, e.g., Liang *et al.*, *Science*, 274:1520-1522 (1996) and U.S. Patent 5,593,853), small organic molecule libraries (see, e.g., benzodiazepines, Baum C&EN, Jan 18, page 33 (1993); isoprenoids, U.S. Patent 5,569,588; thiazolidinones and metathiazanones, U.S. Patent 5,549,974; pyrrolidines, U.S. Patents 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent 5,506,337; benzodiazepines, 5,288,514, and the like).

Devices for the preparation of combinatorial libraries are commercially available (see, e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY, Symphony,

Rainin, Woburn, MA, 433A Applied Biosystems, Foster City, CA, 9050 Plus, Millipore, Bedford, MA). In addition, numerous combinatorial libraries are themselves commercially available (see, e.g., ComGenex, Princeton, N.J., Asinex, Moscow, RU, Tripos, Inc., St. Louis, MO, ChemStar, Ltd, Moscow, RU, 3D Pharmaceuticals, Exton, PA, Martek Biosciences, 5 Columbia, MD, etc.).

In one embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the cell or tissue expressing aging associated proteins is attached to a solid phase substrate. In the high throughput assays of the invention, it is possible to screen up to several thousand different modulators or ligands in a single day. In 10 particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 96 modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- about 1500 different compounds. It is possible to assay many 15 plates per day; assay screens for up to about 6,000, 20,000, 50,000, or 100,000 or more different compounds are possible using the integrated systems of the invention.

C. Solid State and soluble high throughput assays

In one embodiment the invention provides soluble assays using aging 20 associated proteins or genes, or a cell or tissue expressing aging associated proteins or genes, either naturally occurring or recombinant. In another embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the aging associated protein or gene is attached to a solid phase substrate.

In the high throughput assays of the invention, it is possible to screen up to 25 several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be used to run a separate assay against a selected potential modulator, or, if concentration or incubation time effects are to be observed, every 5-10 wells can test a single modulator. Thus, a single standard microtiter plate can assay about 100 (e.g., 96) modulators. If 1536 well plates are used, then a single plate can easily assay from about 100- 30 about 1500 different compounds. It is possible to assay many plates per day; assay screens for up to about 6,000, 20,000, 50,000, or more than 100,000 different compounds are possible using the integrated systems of the invention.

The protein of interest, or a cell or membrane comprising the protein of interest can be bound to the solid state component, directly or indirectly, via covalent or non

covalent linkage e.g., via a tag. The tag can be any of a variety of components. In general, a molecule which binds the tag (a tag binder) is fixed to a solid support, and the tagged molecule of interest is attached to the solid support by interaction of the tag and the tag binder.

5 A number of tags and tag binders can be used, based upon known molecular interactions well described in the literature. For example, where a tag has a natural binder, for example, biotin, protein A, or protein G, it can be used in conjunction with appropriate tag binders (avidin, streptavidin, neutravidin, the Fc region of an immunoglobulin, etc.) Antibodies to molecules with natural binders such as biotin are also widely available and
10 appropriate tag binders; see, SIGMA Immunochemicals 1998 catalogue SIGMA, St. Louis MO). Similarly, any haptenic or antigenic compound can be used in combination with an appropriate antibody to form a tag/tag binder pair.

15 Synthetic polymers, such as polyurethanes, polyesters, polycarbonates, polyureas, polyamides, polyethyleneimines, polyarylene sulfides, polysiloxanes, polyimides, and polyacetates can also form an appropriate tag or tag binder. Many other tag/tag binder pairs are also useful in assay systems described herein, as would be apparent to one of skill upon review of this disclosure.

20 Common linkers such as peptides, polyethers, and the like can also serve as tags, and include polypeptide sequences, such as poly gly sequences of between about 5 and 200 amino acids. Such flexible linkers are known to persons of skill in the art. For example, poly(ethylene glycol) linkers are available from Shearwater Polymers, Inc. Huntsville, Alabama. These linkers optionally have amide linkages, sulphydryl linkages, or heterofunctional linkages.

25 Tag binders are fixed to solid substrates using any of a variety of methods currently available. Solid substrates are commonly derivatized or functionalized by exposing all or a portion of the substrate to a chemical reagent which fixes a chemical group to the surface which is reactive with a portion of the tag binder. For example, groups which are suitable for attachment to a longer chain portion would include amines, hydroxyl, thiol, and carboxyl groups. Aminoalkylsilanes and hydroxyalkylsilanes can be used to functionalize a variety of surfaces, such as glass surfaces. The construction of such solid phase biopolymer arrays is well described in the literature. See, e.g., Merrifield, *J. Am. Chem. Soc.* 85:2149-2154 (1963) (describing solid phase synthesis of, e.g., peptides); Geysen *et al.*, *J. Immun. Meth.* 102:259-274 (1987) (describing synthesis of solid phase components on pins); Frank & Doring, *Tetrahedron* 44:60316040 (1988) (describing synthesis of various peptide sequences

on cellulose disks); Fodor *et al.*, *Science*, 251:767-777 (1991); Sheldon *et al.*, *Clinical Chemistry* 39(4):718-719 (1993); and Kozal *et al.*, *Nature Medicine* 2(7):753-759 (1996) (all describing arrays of biopolymers fixed to solid substrates). Non-chemical approaches for fixing tag binders to substrates include other common methods, such as heat, cross-linking by 5 UV radiation, and the like.

Another example of a high-throughput assay does not require immobilizing a target protein. Such examples include homogenous assays such as fluorescence resonance energy transfer and fluorescence polarization. Spectroscopy can also be used in a variety of ways. Assays can also be used to generate structure-activity relationships (SAR). A method 10 of analyzing an aging associated protein can also include assays that may not be traditionally associated with a particular throughput, e.g., certain NMR binding assays (e.g., SAR by NMR), calorimetry, crystallization, and so forth.

CELLULAR TRANSFECTION AND GENE THERAPY

15 The present invention provides the nucleic acids of aging associated proteins for the transfection of cells *in vitro* and *in vivo*. These nucleic acids can be inserted into any of a number of well-known vectors for the transfection of target cells and organisms as described below. The nucleic acids are transfected into cells, *ex vivo* or *in vivo*, through the interaction of the vector and the target cell. The nucleic acid, under the control of a promoter, 20 then expresses a protein of the present invention, thereby mitigating the effects of absent, partial inactivation, or abnormal expression of the gene of interest, or increasing lifespan in a subject with normal gene expression. For example, as described herein, overexpression of HSF-1 extends adult lifespan. The compositions are administered to a patient in an amount sufficient to elicit a therapeutic response in the patient. An amount adequate to accomplish 25 this is defined as "therapeutically effective dose or amount."

Such gene therapy procedures have been used to correct acquired and inherited genetic defects. The ability to express artificial genes in humans facilitates the prevention and/or cure of many important human diseases, including many diseases which are not amenable to treatment by other therapies (for a review of gene therapy procedures, see 30 Anderson, *Science* 256:808-813 (1992); Nabel & Felgner, *TIBTECH* 11:211-217 (1993); Mitani & Caskey, *TIBTECH* 11:162-166 (1993); Mulligan, *Science* 926-932 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1998); Vigne, *Restorative Neurology and Neuroscience* 8:35-36 (1995); Kremer & Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada *et al.*, in

Current Topics in Microbiology and Immunology (Doerfler & Böhm eds., 1995); and Yu et al., Gene Therapy 1:13-26 (1994)).

PHARMACEUTICAL COMPOSITIONS AND ADMINISTRATION

5 Pharmaceutically acceptable carriers are determined in part by the particular composition being administered (e.g., nucleic acid, protein, modulatory compounds or transduced cell), as well as by the particular method used to administer the composition. Accordingly, there are a wide variety of suitable formulations of pharmaceutical compositions of the present invention (see, e.g., Remington's Pharmaceutical Sciences, 17th 10 ed., 1989). Administration can be in any convenient manner, e.g., by injection, oral administration, inhalation, transdermal application, or rectal administration.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effective amount of the packaged nucleic acid suspended in diluents, such as water, saline or PEG 400; (b) capsules, sachets or tablets, each containing a 15 predetermined amount of the active ingredient, as liquids, solids, granules or gelatin; (c) suspensions in an appropriate liquid; and (d) suitable emulsions. Tablet forms can include one or more of lactose, sucrose, mannitol, sorbitol, calcium phosphates, corn starch, potato starch, microcrystalline cellulose, gelatin, colloidal silicon dioxide, talc, magnesium stearate, stearic acid, and other excipients, colorants, fillers, binders, diluents, buffering agents, 20 moistening agents, preservatives, flavoring agents, dyes, disintegrating agents, and pharmaceutically compatible carriers. Lozenge forms can comprise the active ingredient in a flavor, e.g., sucrose, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and the like containing, in addition to the active ingredient, carriers known in the art.

25 The compound of choice, alone or in combination with other suitable components, can be made into aerosol formulations (i.e., they can be "nebulized") to be administered via inhalation. Aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and the like.

30 Formulations suitable for parenteral administration, such as, for example, by intraarticular (in the joints), intravenous, intramuscular, intradermal, intraperitoneal, and subcutaneous routes, include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain antioxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers,

and preservatives. In the practice of this invention, compositions can be administered, for example, by intravenous infusion, orally, topically, intraperitoneally, intravesically or intrathecally. Parenteral administration and intravenous administration are the preferred methods of administration. The formulations of compounds can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials.

5 Injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described. Cells transduced by nucleic acids for *ex vivo* therapy can also be administered intravenously or parenterally as described above.

10 The dose administered to a patient, in the context of the present invention should be sufficient to effect a beneficial therapeutic response in the patient over time. The dose will be determined by the efficacy of the particular vector employed and the condition of the patient, as well as the body weight or surface area of the patient to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that accompany the administration of a particular vector, or transduced cell type in a 15 particular patient.

In determining the effective amount of the vector to be administered in the treatment or prophylaxis of conditions owing to diminished or aberrant expression of the protein of choice, the physician evaluates circulating plasma levels of the vector, vector toxicities, progression of the disease, and the production of anti-vector antibodies. In general, 20 the dose equivalent of a naked nucleic acid from a vector is from about 1 µg to 100 µg for a typical 70 kilogram patient, and doses of vectors which include a retroviral particle are calculated to yield an equivalent amount of therapeutic nucleic acid.

For administration, compounds and transduced cells of the present invention can be administered at a rate determined by the LD-50 of the inhibitor, vector, or transduced 25 cell type, and the side-effects of the inhibitor, vector or cell type at various concentrations, as applied to the mass and overall health of the patient. Administration can be accomplished via single or divided doses.

It is understood that the examples and embodiments described herein are for 30 illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims. All publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

The following examples are provided by way of illustration only and not by way of limitation. Those of skill in the art will readily recognize a variety of noncritical parameters that could be changed or modified to yield essentially similar results.

Example 1: Objective characteristics of aging identified using Nomarski differential interference contrast microscopy

A. *Introduction*

In this study, the tissues of wild-type animals were compared to those of long-lived and short-lived insulin/IGF-1 signaling mutants during the course of their lives. We have found that Nomarski differential interference contrast (DIC) microscopy provides an effective, rapid and convenient means of visualizing many features of tissue aging. Using this method, we have found that extensive tissue deterioration takes place during aging, not only in the post-mitotic somatic tissues of the animal, but also in a mitotic lineage, the germline. Our findings indicate that insulin/IGF-1 signaling influences lifespan by changing the rate at which the tissues age, and that this pathway governs the aging not only of the post-mitotic somatic cells, but mitotic lineages as well. Interestingly, the majority of animals we have examined whose adult lifespans were shortened by mutations or certain RNAi treatments did not look prematurely "old" upon observation with Nomarski microscopy. However, animals with lifespan mutations that caused shorter lifespans due to premature aging looked prematurely old (e.g., *hsp*). Thus, this technique aids in distinguishing mutated animals who die young, and mutated animals with premature aging, even though both classes of animals have shortened adult lifespans.

25

B. *Methods*

The following strains were used: N2, *daf-16(mu86)I*, *daf-2(e1370)III*, *daf-2(mu150)III*, *daf-2(e1370)III*; *deg-1(u38)X*, *ced-3(n1286)IV*, *DH1033 bIs1[vit-2::gfp; rol-6]; sqt-1(sc103)*, *daf-2(e1370)III*; *him-5(e1490)V*.

30 **Lifespan Analysis:** Wild-type, *daf-2* and *daf-16* animals raised at 20°C were shifted to 25°C at the L4 molt, and transferred to new plates every other day thereafter until progeny production ceased. Animals were judged to be dead when they no longer responded to gentle prodding. Animals that crawled off the plate, became desiccated on the sides of the plate, displayed extruded internal organs or died from internally-hatched progeny were

censored. Censored animals were incorporated into the data set until the day of their disqualification, as described previously (Apfeld & Kenyon *Nature* 402:804-9 (1999)). Lifespan analysis of *ced-3* mutant animals was conducted at 25°C using isogenic N2 from the Horvitz lab, kindly provided by Cori Bargmann, as a control. Lifespans of *daf-2*; *deg-1* animals were assayed at 20°C. Statview 5.0.1 (SAS) software was used to construct lifespan curves, and to determine means and percentiles. Ages given refer to days of adulthood.

5 **Lipofuscin:** Endogenous gut fluorescence was photographed using a 525 nm bandpass filter. Images were collected without automatic gain control in order to preserve the relative intensity of different animals' fluorescence. 2-, 5- and 10-day old adults were
10 photographed on the same day in order to avoid effects of light source variation on apparent fluorescence intensity.

15 **Visualization of Yolk:** The *vit-2::GFP* fusion strain (see above) was a kind gift of David Hersch and Barth Grant. Animals were allowed to age and were photographed using both Nomarski optics and epifluorescence (525 nm).

20 **Nomarski Analysis:** Animals were placed on a 2% agarose pad in M9 buffer with 2mM sodium azide and covered with a coverslip. Control experiments indicated that sodium azide did not affect the age-related phenotypes we observed. Delicate older animals of all genotypes occasionally ruptured and were lost during this process (approximately 10%). Images were captured using a CCD camera coupled to Universal Imaging Corporation's MetaMorph Imaging System (version 3.6). Image files were contrast-balanced and rotated when necessary using PhotoShop 5.0.

25 **Quantification of Tissue Damage:** *C. elegans* heads were photographed as described above. In a blind experiment, photographs of heads were given a score of 1-5, with 1 representing a youthful, unsullied appearance, and 2 through 4 denoting low, medium and high levels of overall deterioration. A rare score of 5 was assigned to animals so deteriorated as to be nearly unrecognizable.

30 Photographs of germ cells in the distal gonad also were rated on a scale of 1-5 based on these criteria. In addition, these photographs were also assigned a cumulative value that represented the presence and extent of several of the correlates of aging, e.g., graininess; large, well-separated nuclei; cavities; and a shriveled appearance. Each correlate of aging was given a separate score, which contributed to the final score. Animals could thus be compared not only by the extent of age-related degeneration, but also by the type of changes they exhibited.

Scores were assigned without knowledge of the age or genotype of the worm in the photograph. Overall scores were re-evaluated at least once, and a naive observer was asked to score a selection of photographs in a double-blind experiment.

Statistical Analysis of Tissue damage: Nonparametric analysis of head
5 scores was conducted, using the Kruskal-Wallis Test to determine if there were significant differences between multiple groups, followed by a pairwise comparison, the Mann-Whitney test. All statistical analysis was conducted using Statview 5.0.1 (SAS) software.

C. Results

10 Decline of tissue integrity in aging wild-type animals

Nomarski optics are commonly used to observe the development of *C. elegans*. In young animals, the nuclei and nucleoli of all cells are readily visualized using this method. It is also possible to see the boundaries of certain cells and tissues, such as the muscles, gonad, epidermal seam cells, and certain neurons. We observed that in young adults, the cells and tissues appeared similar to those of late juvenile stages, except that the nuclear boundaries were less distinct. This lack of definition became more pronounced as the animals grew older. For example, by day 10 of adulthood, it was very difficult to see the nuclei of the epidermal cells. Neuronal nuclei, which have a wrinkled, "raisin-like" appearance, remained visible throughout the life of the animal, although they, too, grew less distinct with time. In young animals, the cytoplasm and nucleoplasm of most cells are smooth and uniform. However, as the animals grew older, both began to show signs of deterioration. Necrotic cavities of various sizes appeared, often containing vibrating particles that appeared to display Brownian motion. Tissues often acquired a curdled texture.

In older animals, the pharynx, as well as the anterior and anal regions of the intestine were frequently distended and packed with the bacteria. We wondered whether these animals were failing to chew or expel their food, or whether they might be succumbing to bacterial infection. To test this, we looked for constipation in animals grown on plates seeded with bacteria killed by UV-irradiation. We found that worms grown on killed bacteria also become highly constipated shortly before death. This suggests that very aged animals lose the ability to chew or expel their food.

30 Older animals also accumulated shiny, mobile patches of a substance that appeared be yolk. In young animals, yolk is transported from its site of synthesis in the intestine into the gonad, where it is incorporated into embryos. It is possible that yolk accumulates in old animals when the production of embryos ceases. We examined animals

expressing a GFP-tagged yolk protein, and confirmed that this substance was in fact yolk. We also observed shiny but less-mobile material in the bodies of worms that did not show GFP fluorescence. Finally, we also observed increased intestinal autofluorescence, which is thought to be caused by lysosomal deposits of lipofuscin. Lipofuscin is a pigment that progressively accumulates in aging eukaryotic tissues as a result of the oxidative degradation and autophagocytosis of cellular components. Its ubiquitous occurrence in a variety of organisms makes it a universal marker of aging.

The cellular deterioration that we observed during aging was widespread. We chose to study the head of the worm in more detail, since it is a particularly informative and compact area composed of multiple tissue types. The head contains the pharynx, a neuromuscular pump that ingests and grinds bacteria, as well as nervous tissue, muscle, and surrounding epidermis. Using Nomarski optics, we were able to evaluate the general character of this body region, but, because cellular boundaries are often indistinct, we were not able to resolve individual tissue types with certainty.

To quantify the changes we observed, we analyzed photographs of 84 individual wild-type worm heads and assigned each animal a numerical value that represented the extent of damage and deterioration it exhibited (see Methods). At every age, some animals exhibited more extensive tissue deterioration than others, consistent with the fact that some animals live longer than others. Nevertheless, the average level of tissue deterioration in the overall population increased steadily with age in a statistically-significant manner. Together these findings indicate that the tissues of *C. elegans* deteriorate in a progressive fashion as the animals grow older. This tissue decline can account for the decreased mobility and the flaccid appearance of old worms that are visualized with a low power-dissecting microscope, and is likely to contribute to the death of the animals.

Tissue deterioration occurs in the germline of aging wild-type animals

The only cells that are able to divide in *C. elegans* adults are the germline stem cells. Thus it was particularly interesting to ask whether signs of age-related tissue deterioration were present in this tissue. We found that the germlines of older animals showed dramatic signs of aging. The germline of *C. elegans* is a multinucleate syncytium; however, in young animals the boundaries of individual nuclei are easy to see. We found that older animals often had fewer nuclei within the gonad, that the boundaries of these nuclei were often ragged. In addition, the surrounding tissue often acquired a granular texture. Occasionally the nuclei were enlarged and appeared to be cellularized, suggesting that they

might have entered meiosis. These changes became apparent at approximately the fifth day of adulthood and increased with age. Our findings indicate that integrity of the germline declines during the life of the animal. Germline deterioration was quantified as described above, by assigning photographs of individual animals a value that reflected the condition of the tissue. Two-day, five-day and ten-day-old animals exhibited progressively more extensive degeneration, and these differences were statistically significant ($p = .0003$).
5

Mutations in the insulin/IGF-1 pathway change the rate at which both mitotic and post-mitotic tissues age

10 To ask how mutations in the insulin/IGF-1 signaling system influence tissue aging, we examined long-lived *daf-2(e1370)* and *daf-2(mu150)* mutants and short-lived *daf-16(mu86)* mutants at different ages. The *daf-2(e1370)* allele has been characterized previously (Dorman *et al.*, *Genetics* 141:1399-1406 (1995); Gems *et al.*, *Genetics* 150:129-155 (1998); Larsen *et al.*, *Genetics* 139:1567-1583 (1995)). At 25°, this mutant is
15 uncoordinated and produces progeny late in life (up to day 40; data not shown). It appears dark when viewed with a dissecting microscope. We found that these animals were shorter than normal animals (1.0 vs 1.4 mm, $p < 0.0001$), and their bodies were thinner (47 μ m vs 54 μ m, $p = 0.002$). In contrast, the *mu150* allele, which we isolated in a screen for long-lived
20 mutants, is also long lived but appears much healthier than *e1370*. Unlike *daf-2(e1370)* animals, which all become dauers at 25°C, only approximately 30% of *daf-2(mu150)* animals become dauers at this temperature. In addition, *mu150* animals moved normally and did not produce progeny late in life. Their bodies were not dark like *e1370* mutants, but resembled wild type, indicating that fat production and longevity can be uncoupled. They were only slightly shorter than normal (1.2 vs 1.4 mm, $p = 0.01$), and not thinner (53 μ m vs 54 μ m, $p = 0.45$). Overall, this mutant appears remarkably similar to wild type in terms of its behavior
25 and morphology. We also examined short-lived animals carrying the *daf-16* null mutation *mu86*. We were particularly interested to learn whether the signs of aging that we observed in wild-type animals were present in these mutants, or whether they might age in an entirely new way. In addition, since these mutations change the lifespan of the animal, we wanted to know whether they might change the rate at which the tissues aged.
30

We found that the quality of tissue deterioration in both *daf-2* and *daf-16* mutants resembled that of wild type, suggesting that both types of mutants age in a normal way but at a different rate. All of the mutants displayed increased lipofuscin-like intestinal fluorescence at relatively old ages. We found that both the somatic tissues and the germlines

of *daf-2(mu150)* and *daf-16* mutants exhibited age-related damage that appeared identical to that seen in wild-type. *daf-2(e1370)* mutants also aged in a manner similar to wild type, but they exhibited high levels of autofluorescence throughout life, and had less yolk in the body cavity than wild type, even at advanced ages (perhaps because yolk was still exported into progeny). Also, the frequency of constipation and bacterial packing in the pharynx was markedly reduced in *daf-2(e1370)* mutants, although it was present in some infirm individuals.

Although the character of the tissue deterioration we observed in *daf-2* mutants was similar to that of wild type, we found that the rate of this deterioration was dramatically slowed. For example, in *daf-2(e1370)* mutants, the outlines of epidermal nuclei were clearly visible until at least 20 days of adulthood, compared to approximately 5 days in wild type. In addition, it was not until *daf-2* animals were approximately 20 days old that we began to see the cavities and "curdled" tissues that were so common in 5-day old wild-type animals.

We quantified the extent of tissue damage by scoring photographs of individual heads and germ cells in the distal gonad. Nonparametric analysis of scores assigned to *daf-2* and wild type animals of the same age confirmed our impression that *daf-2* animals exhibited far less age-related damage in both tissues. We also examined the tissues of *daf-16* mutants, whose lifespans are slightly shorter than normal at 25° C. In general, *daf-16* animals did not look dramatically older than age-matched wild type animals. On the second day of adulthood, wild type and *daf-16* heads looked marginally older than heads of *daf-2* mutants ($p = 0.04$). By the fifth day of adulthood, the difference in head scores between wild type or *daf-16* and either *daf-2* allele was highly significant ($p < 0.0001$). *daf-16* and wild-type heads and germ cells were indistinguishable at early ages; it was only on the tenth day of adulthood that *daf-16* mutant animals began to look significantly older than wild type when scores of heads ($p = 0.01$) and germ cells ($p = 0.02$) were compared. *daf-16* animals at this age will all be dead within two days, whereas wild type worms have a longer life expectancy.

The cavities we saw in old wild-type animals resembled the necrotic cell death seen in certain neurodegeneration mutants, such as the *deg-1* sodium-channel mutants. If *daf-2* mutations delay the appearance of necrotic cavities during aging, they might be expected to suppress or ameliorate these degenerations in *deg-1* animals. However, we found that *daf-2*; *deg-1* double mutants displayed both an abundance of necrotic cavities at a young age and a

long, *daf-2*-like lifespan. This suggests that *daf-2* may not directly suppress necrotic cell death, but instead may act to delay an age-dependent process that causes necrosis.

Apoptosis is unlikely to influence aging in *C. elegans*

In vertebrates, apoptosis, or programmed cell death, occurs throughout life and plays an important role in development and cellular growth control. In *C. elegans*, 131 cells undergo apoptosis, during development. It is reasonable to think that apoptosis, could potentially influence the lifespan of the organism. We looked for apoptotic cells, which have a characteristic refractile appearance (Sulston *et al.*, *Developmental Biology* 56:110-156 (1977)), in the somatic tissues of wild type and mutant animals during aging, and failed to observe them. Apoptotic cell death does occur in early meiotic cells of the germline (Gumienny *et al.*, *Development* 126:1011-1022 (1999)). To test the role of apoptosis in a more definitive way, we examined the lifespans of *ced-3* mutants, which lack a caspase that is required for apoptosis in *C. elegans*. In *ced-3* mutants, cells that should die instead remain alive and, at least in some cases, differentiate into functional cells. We reasoned that if programmed cell death influences organismal death, mutants defective in apoptosis should have abnormal, possibly extended, lifespans. However, we found that *ced-3* mutants had lifespans that were indistinguishable from those of wild type. We conclude that apoptosis does not play a significant role in the regulation of wild-type lifespan.

20

Example 2: *C. elegans* mutants that extend lifespan

Using libraries representing double stranded RNAs from Chromosome I (Fraser *et al.*, *Nature* 408:325-330 (2000)) and Chromosome II, genes that extend the lifespan of *C. elegans* when inhibited were identified. The gene encoded include mitochondrial respiratory proteins, glycolytic proteins, a GTPase, and three genes of unknown function.

25

A. Screen for Lifespan Enhancing Genes.

Animals were cultured on bacterial strains expressing double stranded RNA in a bacterial feeding library. (Kamath *et al.*, *Genome Biol.* 2:research0002.1-0002.10 (2000); Fraser *et al.*, *Nature* 408:325-330 (2000)). The library was made as follows. The sequence of the *C. elegans* genome is known and has been used to identify predicted genes. DNA fragments corresponding to predicted genes were cloned into a feeding vector L4440 between two T7 bacterial promoters in inverted orientation. The library was then transformed into a

bacterial strain carrying IPTG-inducible expression of T7 polymerase. Expression of the dsRNA was induced by addition of IPTG.

5. *C. elegans* were cultured on bacterial strains expressing dsRNA corresponding to predicted gene sequences from Chromosomes I or II. Worm lifespan was monitored. A sterile *C. elegans* strain (fem-1, fer-2 double mutant) was used to allow the lifespan of a single generation to be analyzed. To analyze lifespan, after reaching adulthood worms were transferred to new plates at least once every seven days. Worms were scored as dead if they failed to respond to being prodded three times with a wire loop. Lifespan data was entered into the Statview statistical data management program for analysis.

10

B. *Inhibiting Metabolic Pathways Results in Enhanced Lifespan.*

15 In the present invention, several metabolic genes were identified that influence the lifespan of *C. elegans* in an RNAi screen of genes located on chromosome I. All of the genes on chromosome I were expressed as double-stranded RNA in a bacterial feeding library (see, e.g., Fraser et al., *Nature* 408:325-330 (2000)). *C. elegans* were cultured on each of these bacterial strains, and lifespan was monitored. Inhibition of any of four metabolic genes extended lifespan significantly.

Respiratory chain mutants

20 During a systematic screen of a *C. elegans* Chromosome I RNAi library it was discovered that animals grown on bacteria expressing *atp-3* double-stranded RNA lived much longer than normal (Fraser et al., *Nature* 408:325 (2000)). Lifespan analysis was conducted at 25°C as described previously (Kenyon et al., *Nature* 366:461 (1993)). The pre-fertile period of adulthood was used as t=0 for lifespan analysis. Strains were grown at 20°C for at 25 least two generations before use in lifespan analysis. Statview 5.0.1 (SAS) software was used for statistical analysis and to determine means and percentiles. In all cases, *p* values were calculated using the logrank (Mantel-Cox) method.

atp-3 encodes a component of the mitochondrial ATP synthase (Complex V). In addition, we found that RNAi of three genes encoding components of the mitochondrial respiratory chain also extended lifespan. These were *nuo-2*, which encodes a component of complex I (NADH/ubiquinone oxidoreductase), *cyc-1*, which encodes a component of complex III (cytochrome C reductase) and *cco-1*, which encodes a component of complex IV (cytochrome C oxidase). Results are shown in Table 1.

Table 1
Statistical analysis of adult lifespans

<u>Strain/treatment</u>	<u>Mean ± s.e.m. (days)</u>	<u>75th percentile (days)*</u>	<u>Number of animals that died/total†</u>	<u>P</u>
N2 animals grown at 25°C on bacteria containing:				
Vector (α)	15.2 ± 0.4	18	48/52	
<i>daf-2</i> dsRNA	20.1 ± 0.9	26	55/71	<0.0001\$
Complex I (<i>nuo-2</i>) dsRNA	21.6 ± 0.9	25	51/54	<0.0001\$
Complex IV (<i>cco-1</i>) dsRNA	24.5 ± 0.7	27	67/69	<0.0001\$
Complex V (<i>atp-3</i>) dsRNA	22.1 ± 0.9	30	54/54	<0.0001\$
Vector (β)	13.6 ± 0.5	17	65/69	
Complex III (<i>cyc-1</i>) dsRNA	25.4 ± 0.9	29	65/69	<0.0001¢
<i>gpi-1</i> dsRNA	18.2 ± 0.5	22	63/67	<0.0001¢
Antimycin A Treatments				
<i>fer-15(b26); fem-1(hc17)</i> + ethanol	16.6 ± 0.6	20	57/61	
<i>fer-15(b26); fem-1(hc17)</i> + 0.1 μ M Antimycin A	20.4 ± 0.7	25	51/61	<0.0001‡
Lifespan of <i>fer-15(b26); fem-1(hc17)</i> animals grown at 25°C on bacteria containing the following dsRNA for the indicated times:				
Vector, larval and adult	16.6 ± 0.6	20	57/64	
Complex III (<i>cyc-1</i>) dsRNA, larval and adult	24.0 ± 1.0	29	50/68	<0.0001△
Complex III (<i>cyc-1</i>) dsRNA, adult only	16.9 ± 0.6	20	62/63	0.6585△
Complex V (<i>atp-3</i>) dsRNA, larval and adult	25.2 ± 0.8	29	61/80	<0.0001△
Complex V (<i>atp-3</i>) dsRNA, adult only	15.7 ± 0.5	18	55/64	0.1714△
<i>gpi-1</i> dsRNA, larval and adult	20.2 ± 0.8	25	68/71	<0.0001△
<i>gpi-1</i> dsRNA, adult only	16.3 ± 0.7	20	61/63	0.6075△
<i>daf-16(mu86)</i> animals grown at 25°C on bacteria containing:				
Vector (α)	9.8 ± 0.2	11	45/47	
<i>daf-2</i> dsRNA	9.7 ± 0.2	10	55/62	0.0792#
Complex I (<i>nuo-2</i>) dsRNA	13.7 ± 0.3	15	59/60	<0.0001#
Complex IV (<i>cco-1</i>) dsRNA	14.6 ± 0.4	18	60/60	<0.0001#
Complex V (<i>atp-3</i>) dsRNA	14.5 ± 0.6	18	35/37	<0.0001#
Vector (β)	9.2 ± 0.3	10	60/69	
Complex III (<i>cyc-1</i>) dsRNA	16.1 ± 0.3	17	43/53	<0.0001∞
<i>gpi-1</i> dsRNA	10.8 ± 0.3	12	57/64	<0.0001∞

<i>daf-2(e1370)</i> animals shifted to 25°C as L4 and grown on bacteria containing:				
Vector	25.1 ± 1.2	34	55/58	
Complex I (<i>nuo-2</i>) dsRNA	48.5 ± 1.4	54	47/60	<0.0001 [¶]
Complex III (<i>cyc-1</i>) dsRNA	41.5 ± 1.8	54	52/60	<0.0001 [¶]
Complex IV (<i>cco-1</i>) dsRNA	48.3 ± 1.4	56	50/62	<0.0001 [¶]
Complex V (<i>atp-3</i>) dsRNA	40.8 ± 3.2	54	46/59	<0.0001 [¶]
<i>gpi-1</i> dsRNA	34.5 ± 1.0	39	58/60	<0.0001 [¶]
Alteration of the reproductive system of N2 animals grown at 20°C on bacteria containing:				
intact + vector	22.3 ± 0.5	24	62/80	
intact + Complex IV (<i>cco-1</i>) dsRNA	28.9 ± 1.4	38	63/80	<0.0001 [¶]
germline ablation + Complex IV (<i>cco-1</i>) dsRNA	44.8 ± 1.9	53	44/46	<0.0001 [¶]
whole-gonad ablation + Complex IV (<i>cco-1</i>) dsRNA	31.7 ± 1.5	40	41/44	<0.0001 [¶]
Alteration of the reproductive system of <i>clk-1(qm30)</i> animals grown at 20°C:				
N2	19.8 ± 0.5	24	132/159	
<i>clk-1(qm30)</i>	31.0 ± 1.2	37	67/80	<0.0001 [¶]
<i>clk-1(qm30)</i> germline ablation	37.5 ± 2.4	49	49/55	<0.0001 [¶]
<i>clk-1(qm30)</i> whole-gonad ablation	19.9 ± 0.8	23	45/50	0.4549 [¶]

* The 75th percentile is the age when the fraction of animals alive reaches 0.25.

† The total number of observations equals the number of animals that died plus the number censored. Animals that crawled off the plate, exploded or bagged were censored at the time of the event. This step incorporated those worms into the data set until the censor date, and was necessary to avoid the loss of information; for example, if a 50-day-old animal crawls off the plate, it is important to include that information in the data set, as that animal was long lived. Control and experimental animals were cultured in parallel and plates were changed at the same time. The logrank (Mantel-Cox) test was used to test the hypothesis that the survival functions among groups were equal. *P* values were calculated for individual experiments, each consisting of control and experimental animals examined at the same time.

§ Compared with N2 worms cultured on HT115 bacteria containing an empty plasmid vector at 25°C. Vector (α), *daf-2*, Complex I, IV and V dsRNA experiments were conducted at the same time.

¶ Compared with N2 worms cultured on HT115 bacteria containing an empty plasmid vector at 25°C. Vector (β), Complex III, and *gpi-1* dsRNA experiments were conducted at the same time.

‡ Compared with control *fer-15(b26); fem-1(hc17)* double mutants grown on equivalent amount of solvent (ethanol) at 25°C.

5 Δ Compared with control *fer-15(b26); fem-1(hc17)* double mutants cultured on bacteria containing an empty plasmid vector at 25°C.

Compared with *daf-16(mu86)* mutants cultured on bacteria containing an empty plasmid vector at 25°C. Vector (α), *daf-2*, Complex I, IV and V dsRNA experiments were conducted at the same time.

10 ∞ Compared with *daf-16(mu86)* mutants cultured on bacteria containing an empty plasmid vector at 25°C. Vector (β), Complex III and *gpi-1* experiments were conducted at the same time.

15 ¶ Compared with *daf-2(e1370)* mutants cultured on bacteria containing an empty plasmid vector shifted to 25°C as L4 larvae.

¥ Compared to N2 worms with an intact reproductive system cultured on bacteria containing an empty plasmid vector at 20°C.

20 √ Compared to N2 worms with an intact reproductive system cultured on OP-50 bacteria at 20°C.

The protein ATP3 (Genbank # AAB37654.1) is a member of the ATP synthase delta family and is 43% identical to human ATP50. NUO2 (Genbank # AAB52474.1) protein is 56% identical to human NDUFS3. CYC1 (Genbank # CAA99820.1) is 50% identical to human CYC1. CCO1 (Genbank # CAB03002.1) is 35% identical to human COX5B. GPI-1 (Genbank # CAB60430.1) is 68% identical to human GPI.

30 We also found that treatment of wild-type animals with the drug antimycin A, which inhibits complex III (Slater, *Trends Biochem Sci.* 8:239 (1983)), increased lifespan as well.

OP-50 bacteria were spread onto NG plates and allowed to grow for two days at room temperature. 50 µl of 0.01 M Antimycin A (Sigma) dissolved in 100% ethanol was dropped onto bacterial lawn and allowed to dry overnight in a fume hood at room temperature. Eggs of *fer-15(b26); fem-1(hc17)* double mutants, which become sterile adults at 25°C, were added to the dried plates and incubated at 25°C. Animals were transferred to new Antimycin A plates every 4 days. Control worms (ethanol only) were handled in the same manner (Table 1).

Because RNAi reduces the level of wild-type mRNA, these treatments were all predicted to decrease the rate of electron transport and ATP production. We measured ATP levels as follows. For each time point, 750 *fer-15(b26); fem-1(hc17)* double mutants were grown at 25°C, to inhibit progeny production. Each time point was repeated in triplicate. The collected worms were washed four times with S-basal buffer, resuspended in cell lysis buffer and quickly frozen in liquid N₂. All samples were stored at -80°C and processed on the same day. A Roche ATP Bioluminescent HSII kit was used to measure ATP concentrations with an OPTOCOMP I luminometer. ATP concentrations were normalized to absolute protein concentrations. A BioRad protein assay kit was used to measure protein concentrations using a Perkin Elmer MBA2000 spectrophotometer.

ATP levels were reduced 60-80% in animals subjected to complex III (*cyc-1*) or ATP synthase (*atp-3*) RNAi, and 40-60% in animals treated with complex I (*nuo-2*) or complex IV (*cco-1*) RNAi. Thus, these perturbations all decrease the rate of mitochondrial respiration.

In addition to extending lifespan, respiratory-chain RNAi affected growth and behavior. First, the animals were smaller than normal, suggesting that mitochondrial respiration promotes growth. Because the small animals were well proportioned, it seems likely that a metabolite whose level is regulated by mitochondrial respiration acts as a signal to control body size. Small body size itself is unlikely to cause lifespan extension, because mutants defective in *daf-4*, which encodes a Smad protein (Estevez *et al.*, *Nature* 365:644 (1993)), are small but not long lived (Tissenbaum *et al.*, *Nature* 410:227 (2001)). Respiratory-chain RNAi also decreased the rate of growth to adulthood, as well as the rates of pharyngeal pumping (eating) and defecation (Table 2). In addition, the animals moved much more slowly than normal (Table 2). Mutations in *mev-1*, *gas-1*, *clk-1* and *isp-1* all reduce the rates of development and movement (Ishii *et al.*, *Nature* 394:694 (1998); Hartman *et al.*, *Mech Aging Dev.* 122:1187 (2001); Wong *et al.*, *Genetics* 139:1247 (1995); Feng *et al.*, *Developmental Cell* 1:663 (2001)) but do not affect body size, suggesting that the level of respiratory activity in our RNAi-treated animals may be lower than in these mutants.

Table 2
Timing of biological processes in RNAi-treated and *clk-1* mutant animals

dsRNA or Other Treatment	Feeding Rate (pumps/min. ± s.d.)*	Defecation Rate (seconds/defecation ± s.d.)†	Mobility (swim strokes/min. ± s.d.)‡	Length of post-embryonic development (hours ± s.d.)‡
Vector	201 ± 9	41 ± 3	130 ± 6	43 ± 2
Complex I(<i>nuo-2</i>)	159 ± 8	80 ± 4	90 ± 2	69 ± 3
Complex III(<i>cyc-1</i>)	161 ± 10	81 ± 1	75 ± 13	88 ± 2
Complex IV(<i>cco-1</i>)	144 ± 9	79 ± 2	94 ± 3	68 ± 2
Complex V(<i>atp-3</i>)	124 ± 17	87 ± 5	90 ± 9	96 ± 2
<i>gpi-1</i>	198 ± 8	43 ± 3	127 ± 6	43 ± 2
<i>clk-1(qm30) intact</i>	161 ± 36	69 ± 6	77 ± 9	
<i>clk-1(qm30) germline-ablated</i>	140 ± 20	50 ± 8	91 ± 7	
<i>clk-1(qm30) gonad-ablated</i>	117 ± 22	63 ± 9	93 ± 5	

* Mean ± s.d. The number of pharyngeal pumps observed in a one-day-old adult animal in 1 minute at 20°C (n ≥ 8).

† Mean ± s.d. The time required to complete one defecation cycle. A minimum of three consecutive defecations was recorded for each trial at 20°C (n ≥ 5).

Mean ± s.d. The number of swim cycles completed in 1 minute of a one-day-old adult animal at 20°C (n ≥ 5).

‡ Mean ± s.d. Time (hours) to adulthood at 20°C (n ≥ 25).

Because RNAi can be administered at different times during the life of an animal, we were able to ask whether mitochondrial respiration acts in an ongoing fashion throughout life to influence aging. To do this, we transferred animals onto bacteria expressing respiratory-chain dsRNA at different ages (Dillin *et al.*, *Nature*, submitted).

Surprisingly, we found that if we allowed the animals to grow to adulthood on normal bacteria, and then shifted them as young adults onto bacteria expressing respiratory-chain dsRNA, their lifespans were not extended (Table 1). To determine whether RNAi lowered respiratory-chain activity in these animals, we assayed their ATP levels and found that they were reduced to the same extent as in animals exposed to respiratory-chain dsRNA from hatching. Thus respiratory-chain RNAi does decrease mitochondrial activity in adult

animals. Together these findings indicate that the respiratory chain acts during development and not during adulthood to influence lifespan.

We also asked whether initiating RNAi during adulthood decreased behavioral rates. The rate of movement we observed was the same as that of control animals, but the 5 rate of pumping was decreased (Table 3). Interestingly, however, whereas animals treated with RNAi from the time of hatching pumped in a slow but steady fashion, animals treated as young adults exhibited bursts of rapid pumping followed by pauses. Possibly these pauses were caused by transient depletion of ATP.

10

Table 3
Pumping rates of larva and adults treated with respiratory-chain RNAi

RNAi Treatment	Feeding Rate (pumps/min ± s.d.)*			
	Day 2	Day 3	Day 4	Day 5
Vector	118 ± 15 (0/8)	140 ± 21 (0/8)	145 ± 24 (0/8)	131 ± 49 (1/10)
Complex III (<i>cyc-1</i>) larval + adult	97 ± 21 (0/10)	83 ± 12 (0/10)	84 ± 12 (0/10)	75 ± 12 (0/10)
Complex III (<i>cyc-1</i>) adult only	79 ± 62 (5/12)	112 ± 80 (3/12)	80 ± 59 (4/12)	69 ± 45 (4/12)
Complex V (<i>atp-3</i>) larval and adult	98 ± 15 (0/10)	87 ± 10 (0/10)	80 ± 9 (0/10)	75 ± 8 (0/10)
Complex V (<i>atp-3</i>) adult only	88 ± 80 (6/14)	98 ± 64 (5/14)	83 ± 59 (4/14)	89 ± 54 (4/14)

* Mean ± s.d. The number of pharyngeal pumps observed in an adult animal in 1 minute at 25°C. Number in parentheses is the number of animals that produced less than 50 pumps per 15 minute/ total number of animals tested.

Interaction of respiratory chain mutants with the DAF 16 pathway.

The insulin/IGF-1 signaling pathway affects lifespan (Guarente *et al.*, *Nature* 408:25 (2000)). Reducing the activity of DAF-2, an insulin/IGF-1 receptor homolog, or 20 downstream signaling components, extends lifespan approximately two-fold. This lifespan extension requires activity of the forkhead-family transcription factor DAF-16.

However, we found that *daf-16(mu86)* null mutants lived much longer than normal when subjected to respiratory-chain RNAi (Table 1). In addition, we found that the already long lifespans of *daf-2(e1370)* mutants were nearly doubled again when the animals 25 were cultured on any of the four bacterial strains expressing respiratory chain dsRNA (Table 1). Moreover, unlike reduction of respiratory chain activity, reduction of insulin/IGF-1

signaling is known to cause a significant increase in ATP levels (Braeckman *et al.*, *Curr Biol*, 9:493 (1999)).

Finally, both *daf-2* and *daf-16* act exclusively in adults to regulate lifespan (Dillin *et al.*, *Nature*). Together these findings indicate that respiratory-chain RNAi does not 5 increase lifespan by inhibiting the DAF-2 pathway.

Interaction of respiratory chain mutants with the reproductive pathway.

In *C. elegans*, removing the germline extends lifespan (Hsin *et al.*, *Nature* 399:362 (1999)). This lifespan extension is not caused by sterility *per se*, because animals in 10 which the somatic gonad as well as the germline are removed have normal lifespans (Hsin *et al.*, *Nature* 399:362 (1999); Kenyon *et al.*, *Nature* 366:461 (1993)).

We found that animals treated with respiratory-chain RNAi were either sterile or had small broods. The longevity of these animals was not likely to be caused by reduced 15 germline activity, however, because the lifespan extension caused by germline ablation is *daf-16* dependent (Hsin *et al.*, *Nature* 399:362 (1999)).

To test whether the reproductive system could influence lifespan in these RNAi-treated animals, we removed either their germlines or the entire reproductive systems. The germlines or the entire reproductive systems of complex IV (*cco-1*) RNAi-treated 20 animals were removed by killing Z2 and Z3 or Z1 and Z4, respectively. Laser ablations were performed on newly hatched animals using a nitrogen-pumped dye laser, which produces a wavelength of 440 nm. Successful ablation was confirmed by examining the reproductive systems of adult animals with a dissecting microscope. Worms were anaesthetized with 1.0 - 1.5 M NaN₃ during ablation, a treatment that had no effect on lifespan. The germline and 25 whole-gonad of ablation *clk-1(qm30)* mutants was performed as described above, in two experiments, with similar results.

In addition to the laser ablation experiments described above, the germlines of respiratory-chain RNAi-treated animals were ablated genetically using *glp-1(e2141ts)* mutant worms, which, at 25°C, produce only a few germ cells and live longer than wild type. Mean 30 lifespans of *glp-1(e2141ts)* mutant worms grown at 25°C on bacteria expressing the following dsRNA were: Control (empty vector), 15.0 ± 0.7; Complex I (*nuo-2*) dsRNA, 19.2 ± 0.7 (*p* <0.0001); Complex III (*cyc-1*) dsRNA, 19.7 ± 1.0 (*p* <0.0001); Complex IV (*cco-1*) dsRNA, 22.9 ± 0.6 (*p* <0.0001); and Complex V (*atp-3*) dsRNA, 23.0 ± 0.6 (*p* <0.0001)). As with wild type, germline ablation further extended lifespan, whereas whole-gonad ablation

did not (Table 1). Thus the reproductive system of these animals appeared to influence lifespan in a normal way.

We also examined the role of the reproductive system in *clk-1* mutants. As with animals subjected to respiratory-chain RNAi, ablating the germlines of *clk-1* mutants caused an increase in lifespan. However, removing the whole gonad of *clk-1(qm30)* mutants produced an unexpected result; it completely abolished their lifespan extensions (Table 1). Thus the longevity of these animals is entirely dependent on the presence of the reproductive system. Interestingly, ablation of the gonad did not rescue the slow growth or behavioral phenotypes of *clk-1(qm30)* mutants (Table 2). This finding is significant, because it indicates that the longevity of these mutants is not likely to be caused by their slow rates of growth or behavior.

Glycolytic Pathway Mutants

In our Chromosome I RNAi screen, we also found that lowering the activity of a gene that functions in glycolysis, glucose phosphate isomerase (*gpi-1*), increased lifespan approximately 20% (Table 1). GPI-1 (Genbank # CAB60430.1) is 68% identical to human GPI. The *C. elegans* genome contains only one glucose phosphate isomerase, implying that these animals have lowered rates of glycolysis. Consistent with this, we found that these animals had reduced levels of ATP.

In vertebrates, PGI is required not only for glycolysis but also for the survival of sensory neurons (Chaput *et al.*, *Nature* 332:454 (1988); Faik *et al.*, *Nature* 332:455 (1988)). Sensory neurons are known to influence the lifespan of *C. elegans* (Apfeld *et al.*, *Nature* 402:804 (1999)). However, when we used a fluorescent dye (DiI) to visualize many sensory neurons, we failed to detect any abnormalities (data not shown). In addition, whereas the lifespan extension of sensory mutants is largely *daf-16* dependent (Apfeld *et al.*, *Nature* 402:804 (1999)), this was not the case for animals treated with *gpi-1* RNAi (Table 1). Together these findings suggest that *gpi-1* RNAi does not increase lifespan by affecting sensory neurons.

Like respiratory chain RNAi, *gpi-1* RNAi further extended the lifespans of *daf-2(e1370)* mutants (Table 1). Interestingly, unlike animals treated with respiratory-chain RNAi, these animals appeared healthy and were normal in size. They had wild-type rates of growth to adulthood and rates of behavior (Table 2). In addition, animals treated with *gpi-1* RNAi as adults were not long-lived (Table 1), suggesting that, like respiratory-chain activity, *gpi-1* may function during development to affect lifespan.

C. Inhibiting a GTPase Results in Enhanced Lifespan.

Using the screen described above, a Rab-like GTPase was identified from a Chromosome I library. The name of the gene is T23H2.5 and its accession number is 5 AAC482001. Analysis of the mutant demonstrated that inhibition of the gene extended lifespan by 25%. The inhibition of lifespan was independent of the Daf2/16 pathway.

D. Inhibiting *llw* genes results in an enhanced lifespan.

An RNAi screen of a chromosome II library identified three novel genes that 10 extend lifespan when inhibited. *llw-1*, gene Y54G11A.8, contains a TPR-like domain and its accession number is CAA22452 (SEQ ID NO: 7). *llw-3*, gene Y48E1B.1, contains a proline-rich domain and its accession number is CAB07688 (SEQ ID:9). Inhibition of *llw-2*, gene F59E12.10, also resulted in enhanced lifespan, and its accession number is AAB54251 (SEQ ID NO:8). Table 1 shows the result of dsRNA expression corresponding to these genes.

15

Table 4
Inhibition of *llw* genes enhances *C. elegans* lifespan

Gene	dsRNA expressed	% worms dead at day 20
	Vector only	88%
<i>llw-1</i>	Y54G11A.8	
<i>llw-2</i>	F59E12.10	37%
<i>llw-3</i>	Y48E1B.1	52%
<i>llw-4</i>	F45H10.4	35%

Example 3: *C. elegans* mutants that shorten lifespan

20 Using the Chromosome I library described previously, *C. elegans* were fed bacteria expressing dsRNA and screened for shortened lifespan. A cursory examination using objective aging criteria allowed immediate discarding of 6 out of 7 short-lived RNAi-treated strains from further consideration as possible progeric animals. Moreover, the one remaining clone had a dramatic phenocopy of normal aging.

25 The progeric RNAi candidate encoded a homologue of HSF (heat-shock factor), a transcription factor that activates expression of heat-shock genes in response to heat shock and oxidative stress. The HSF-1 gene is Y53C10.12 and the accession number is

CAA22146. As heat shock factor genes are highly conserved, this family of genes therefore is shown to be involved in aging, including human homologs of HSF family genes, e.g., HSF-1.

In a second experiment, over-expression of HSF-1 gene in *C. elegans* caused an increased adult lifespan. The HSF-1 gene has been cloned and constructed into a plasmid that can be used to inject into the worm. The HSF-1 gene was ectopically over-expressed in the worms that carry this plasmid. Compared to wild type worms, these worms have an increased lifespan.

10 Example 4: Screening for genes that affect aging using microarrays

In *C. elegans*, aging is regulated in one instance by an insulin/IGF-1 pathway. An insulin receptor homolog, DAF-2 activates a PI 3 kinase pathway which in turn appears to inhibit the function of DAF-16, a forkhead-family transcription factor. *daf-2* mutants are long lived, whereas *daf-16* and *daf-16; daf-2* mutants are short lived. Previous experiments have shown that this *daf-2* pathway functions non-cell autonomously, meaning that it must regulate the expression of one or more genes that either encode or regulate the production of a downstream signal or hormone. In addition, there must be a cellular pathway that responds to this second hormone, and finally genes that encode proteins directly involved in the aging process.

20 Microarrays were constructed using *C. elegans* DNA primers purchased from Research Genetics. The arrays were then used to analyze gene expression profiles in *daf-2* and *daf-16* mutants. The expression of a number of genes varied in long-lived (*daf-2*) or short-lived (*daf-16* and *daf-16; daf-2*) *C. elegans* mutants (see Table 5). The activity of genes identified using microarray analysis was then inhibited using RNAi (Table 6). The 25 genes thus identified include hormones that activate the *daf-2* pathway, several encoding cytochrome P450 proteins, e.g., genes involved in lipid and steroid biosynthesis, the melatonin synthesis gene (human homolog ASMTL, Accession No. NM_004192), insulin and insulin-like peptides (e.g., ins-7), heat shock factors, catalases, stress-response genes, neuronal genes, oxidative stress protection genes, antibacterial and antifungal genes, 30 vitellogenins, and metabolic genes (see Figures 5-12).

Example 5: Screening for genes and compounds that affect aging using a DAF-16/GFP fusion protein.

A DAF16-GFP fusion was constructed as described in Lin *et al.*, *Nature Gen.* 28:139-145(2001). DAF16 is a transcription factor and in normal animals is localized throughout cells. Using the DAF16-GFP fusion protein, localization of DAF-16 in long lived animals was studied. In animals with extended lifespans, the DAF-16/GFP fusion was localized to the nuclei.

The following extended lifespan mutants were analyzed: *daf-2* mutants and germline ablated animals. DAF-2 is an insulin /IGF-1 receptor homologue. In *daf-2* mutants, lifespan is extended in a *daf-16* dependent manner. When expressed in *daf-2* mutants, the DAF-16/GFP fusion was localized in most nuclei throughout larval development and in adults. In germline ablated animals, the DAF-16/GFP fusion was localized in the nuclei of intestinal cells and to a lesser extent the nuclei of other cell types also.

Localization of DAF-16/GFP is used to screen for drugs or mutations that extend lifespan. Localization of DAF-16/GFP is also used to determine whether a mutation extends lifespan by perturbing the insulin/IGF-1 pathway, germline pathway, or other aging regulatory pathway.

Example 6: Screening for genes and compounds that affect aging using an SOD-3/GFP fusion protein

The *C. elegans* SOD-3 (superoxide dismutase) gene is regulated by the insulin/IGF-1 system. Mutants in the insulin/IGF1 pathway overexpress SOD-3. When an SOD-3/GFP was expressed in long-lived mutants of the insulin/IGF1 pathway, the mutants had a higher level of GFP fluorescence, especially in the intestine, than did wild-type worms expressing the same fusion protein.

Worms expressing the SOD-3/GFP fusion protein are screened for extended lifespan by assaying for intense fluorescence patterns. Screens are done for mutants in the insulin/IGF-1 pathway, for long-lived germline signaling mutants, and for respiratory chain mutants. Screens are also done to identify drugs that affect the aging process.

30

TABLE 5

CF512 WL Sept	description	mean	std err	% vector	% avg V
C04F6.1	vit-5	15.92	0.626	121.5267	103.7134
C17G1.4	muc-1	11.38	0.477	86.87023	74.13681

CF512 WL Sept	description	mean	std err	% vector	% avg V
C17G10.5	Ent.His. N-acetylmuraminidase	13.53	0.846	103.2824	88.14332
C18B2.3		12.22	0.495	93.28244	79.60912
C40H5.1		12.68	0.736	96.79389	82.60586
C44E4.2		13.39	0.79	102.2137	87.23127
C54G4.6	ASMTL	16.92	0.758	129.1603	110.228
C55B7.4	AcylCoADH	13.5	0.885	103.0534	87.94788
Y54G11A.6	ctl-1	16.3	0.748	124.4275	106.1889
Y54G11A.5b	ctl-2	13.53	0.808	103.2824	88.14332
Daf-16		13	0.519	99.23664	84.69055
Daf-2		17.1	0.834	130.5344	111.4007
F08B1.1	vhp-1 phosphatase	15.5	0.644	118.3206	100.9772
F21F3.3	farnesyl cystein carboxyl methyltransferase	12.73	0.501	97.17557	82.9316
F32A5.5	aquaporin	14.37	0.63	109.6947	93.61564
F35H12.2	PTB/PID GAP	14	0.562	106.8702	91.20521
F48D6.4		14.07	1.06	107.4046	91.66124
F56G4.3	F-box domain sim.to pes-2	14.84	0.684	113.2824	96.67752
F59D8.2	vit-4	12.5	0.541	95.41985	81.43322
H16D19.1	C-type lectin	13.22	0.778	100.916	86.12378
H22K11.1	asp-3	15	0.708	114.5038	97.71987
K07A1.7		15.12	0.617	115.4198	98.50163
K10B3.8	gpd-2	14.05	0.679	107.2519	91.53094
K11D2.2	ASAHH acid ceramidase	14.86	1.024	113.4351	96.80782
R03E9.1	mdl-1 tf	15.8	0.522	120.6107	102.9316
T01A4.1	Npr1-like	13.19	0.734	100.687	85.92834
T07D10.4	C-type lectin	12.96	1.03	98.9313	84.42997
T10B9.1	Cyt P450	10.9	0.443	83.20611	71.00977
T25C12.2	like T08A9.9 antibacterial	13.72	0.874	104.7328	89.38111
T25C12.3	EGF/lectin	14.34	1.02	109.4656	93.4202
vector		13.1	0.532	100	85.34202
ZK1320.2		14.63	0.459	111.6794	95.30945
ZK270.2	FERM domain/band 4.1	10.54	0.536	80.45802	68.6645
ZK355.E		15.2	0.585	116.0305	99.0228

CF512adult	description	mean	std err	% vector	% avg V
C04F6.1	vit-5	17.15	0.587	103.1269	111.7263844
C17G1.4	muc-1	18.67	0.411	112.267	121.6286645
C17G10.5	Ent.His. N-acetylmuraminidase	15.724	0.471	94.55201	102.4364821
C18B2.3		15.83	0.544	95.18942	103.1270358
C40H5.1		18.2	0.542	109.4408	118.5667752
C44E4.2		19.33	0.536	116.2357	125.9283388
C54G4.6	ASMTL	18.41	0.803	110.7035	119.9348534
C55B7.4	AcylCoADH	17.27	0.676	103.8485	112.5081433
Y54G11A.6	ctl-1	16.71	0.567	100.4811	108.8599349
Y54G11A.5b	ctl-2	16.38	0.341	98.49669	106.7100977
Daf-16		14.53	0.49	87.37222	94.65798046
Daf-2		21.36	0.953	128.4426	139.1530945

CF512adult	description	mean	std err	% vector	% avg V
F08B1.1	vhp-1 phosphatase	13.43	0.506	80.75767	87.49185668
F21F3.3	farnesyl cystein carboxyl methyltransferase	15.86	0.449	95.36981	103.3224756
F32A5.5	aquaporin	15.43	0.468	92.78413	100.5211726
F35H12.2	PTB/PID GAP	18.87	0.972	113.4696	122.9315961
F48D6.4		17	0.567	102.2249	110.7491857
F56G4.3	F-box domain sim.to pes-2	18.58	0.791	111.7258	121.0423453
F59D8.2	vit-4	17.22	0.657	103.5478	112.1824104
H16D19.1	C-type lectin	15.34	0.541	92.24293	99.93485342
H22K11.1	asp-3	17.36	0.498	104.3897	113.0944625
K07A1.7		15.68	0.559	94.28743	102.1498371
K10B3.8	gpd-2	17.36	0.629	104.3897	113.0944625
K11D2.2	ASAHH acid ceramidase	15.77	0.607	94.82862	102.7361564
R03E9.1	mdl-1 tf	16.96	0.536	101.9844	110.4885993
T01A4.1	Npr1-like	17.25	0.48	103.7282	112.3778502
T07D10.4	C-type lectin	14.93	0.496	89.77751	97.26384365
T10B9.1	Cyt P450	15.83	0.448	95.18942	103.1270358
T25C12.3	like T08A9.9 antibacterial	17.8	0.83	107.0355	115.9609121
vector	EGF/lectin	16.63	0.468	100	108.3387622
ZK1320.2		11.72	0.435	70.47505	76.35179153
ZK270.2		18.3	0.771	110.0421	119.218241
ZK355.E	FERM domain/band 4.1	15.52	0.462	93.32532	101.1074919

CF512 Dec.	description	mean	std err	% vector	% avg. V
AC3.7	UDP-glucuronosyl transferase	14.78	0.365	101.2329	96.28664
B0213.15	Cyt P450	14.35	0.365	98.28767	93.48534
B0554.1		12.67	0.417	86.78082	82.54072
B0554.6	like AK6.11	12.43	0.4	85.13699	80.9772
C04F6.1	vit-5	12.52	0.372	85.75342	81.56352
C17G1.4	muc-1	12.21	0.41	83.63014	79.54397
C17H12.8		15.16	0.325	103.8356	98.76221
C24B9.4	DUF32 domain	14.12	0.376	96.71233	91.98697
C32F10.9		13.59	0.381	93.08219	88.5342
C32H11.10	DUF141	12.92	0.493	88.49315	84.16938
C32H11.12	DUF141	12.1	0.38	82.87671	78.82736
C46F4.2	long-chain fatty acid-Coenzyme A ligase 4	13.33	0.386	91.30137	86.84039
C54D10.1	GST	13.56	0.371	92.87671	88.33876
C54G4.6	ASMTL/Maf-like	12.81	0.422	87.73973	83.45277
C55B7.4	Acyl-CoA DH	13.04	0.431	89.31507	84.95114
Daf-16		8.34	0.288	57.12329	54.33225
Daf-2		16.017	0.235	109.7055	104.3453
F10D2.9	fat-7	14.71	0.33	100.7534	95.83062
F11A5.12	short-chain DH-reductase;hydroxysteroid 17-beta DH	13.93	0.407	95.41096	90.74919
F28D1.3	thaumatin	12.28	0.442	84.10959	80
F28D1.5	thaumatin	12.29	0.463	84.17808	80.06515

F38E11.2	hsp-12.6		14.79	0.383	101.3014	96.35179
F49A5.6	thaumatin		14.43	0.341	98.83562	94.00651
F55G11.5			12.1	0.356	82.87671	78.82736
K04E7.2	pep-2		10.6	0.315	72.60274	69.05537
K06A11.1			15.16	0.374	103.8356	98.76221
K07C6.4	Cyt P450		12.13	0.411	83.08219	79.0228
K10D11.1			14.29	0.337	97.87671	93.09446
K11G9.6	mtl-1		13.16	0.429	90.13699	85.7329
K12G11.3	Alcohol DH		12.58	0.396	86.16438	81.9544
T10B9.1	Cyt P450		10.77	0.309	73.76712	70.16287
T13F2.1	fat-4		13.12	0.369	89.86301	85.47231
T16G12.1	aminopeptidase		14.25	0.367	97.60274	92.83388
T20G5.7			15.7	0.314	107.5342	102.2801
T22G5.2	lbp-7		14.116	0.349	96.68493	91.96091
Vector			14.6	0.372	100	95.11401
W06D12.3	fat-5		13.69	0.429	93.76712	89.18567
W08D2.4	fat-3		12.13	0.241	83.08219	79.0228
Y38H6C.5	Zn knuckle domain		13.25	0.37	90.75342	86.31922
Y49E10.8			15.85	0.291	108.5616	103.2573
ZK1251.2	ins-7		16.4	0.19	112.3288	106.8404
ZK384.4			12.6	0.401	86.30137	82.08469

CF596	description	mean	std err	% vector	CF596	% vector
B0213.15	Cyt P450	26	0.5	111.9242	B0213.15	111.9242
C02A12.4	N-acetylmuraminidase	18.63	0.855	80.19802	vector	100
C05E4.4		18.933	0.913	81.50237	K12G11.3	91.08911
C24B9.4	DUF23	18.59	0.816	80.02583	F10D2.9	82.5226
Daf-16		11.47	0.423	49.37581	C05E4.4	81.50237
Daf-2		13.88	0.659	59.75032	C02A12.4	80.19802
F10D2.9	fat-7	19.17	0.885	82.5226	T20G5.7	80.19802
F28D1.3	thaumatin	18.4	0.927	79.20792	C24B9.4	80.02583
F38E11.2	hsp-12.6	18.42	0.78	79.29402	K11G9.6	79.85364
K11G9.6	mtl-1	18.55	1.02	79.85364	F38E11.2	79.29402
K12G11.3	Alcohol DH	21.16	1.05	91.08911	F28D1.3	79.20792
T10B9.1	Cyt P450	15.36	0.813	66.12139	T10B9.1	66.12139
T20G5.7		18.63	0.938	80.19802	Daf-2	59.75032
vector		23.23	1.02	100	Daf-16	49.37581

rff-3 20C	description	mean	std err	% vector	RNAi	% vector
C04F6.1	vit-5	17.47	0.763	116.4667	daf-2	207.3333
C32H11.12	DUF141	19.7	0.676	131.3333	ZK1251.2	155.2
C42D8.2	vit-2	18.15	0.576	121	C54G4.6	132.38
C54G4.6	ASMTL	19.857	0.658	132.38	C32H11.12	131.3333
daf-16		14.84	0.476	98.93333	ZK896.1	125.4667
daf-2		31.1	0.749	207.3333	C42D8.2	121
vector		15.02	0.613	100.1333	C04F6.1	116.4667
ZK1251.2	ins-7	23.28	0.512	155.2	vector	100.1333
ZK896.8	gcy-18	18.82	0.731	125.4667	daf-16	98.93333

CF512 25	description	mean	std err	% vector	% avg. V
C04F6.1	vit-5	14.7	0.586	86.47059	95.76547
C32H11.12		16.5	0.635	97.05882	107.4919
C42D8.2	vit-2	15.42	0.58	90.70588	100.456
C54G4.6	ASMTL	18.71	0.637	110.0588	121.8893
daf-16		11.2	0.279	65.88235	72.96417
daf-2		18.13	0.76	106.6471	118.1107
vector		17.06	0.574	100.3529	111.1401
ZK1251.2	ins-7	17.05	0.606	100.2941	111.0749
ZK896.8	gcy-18	16.41	0.695	96.52941	106.9055

vector	16.63	0.468
vector	17.06	0.574
Vector	14.6	0.372
vector	13.1	0.532
	15.3475	

TABLE 6

Upregulated in daf-2 mutants and with daf-2 RNAi, downregulated with daf-16 RNAi

Gene	Gene product/homology
C15H9.1	C15H9.1: Protein of unknown function
C53B7.3	C53B7.3: Protein with weak similarity to EGF-like repeats, has moderate similarity to <i>C. elegans</i> F46C8.4
C34C6.7	C34C6.7: Protein of unknown function
E04F6.9	E04F6.9: Protein of unknown function, has moderate similarity to <i>C. elegans</i> E04F6.8
C50F7.5	C50F7.5: Protein of unknown function, has moderate similarity to <i>H. sapiens</i> MUC1 gene product, a transmembrane mucin
Y15E3B.f	Y15E3B.f Y15E3B.f
F09F7.7	F09F7.7: Protein of unknown function
T23G7.3	T23G7.3: Protein of unknown function, has weak similarity to <i>S. cerevisiae</i> Ygr280p
F08B12.4	F08B12.4: Protein of unknown function
F47H4.10	F47H4.10: Member of an uncharacterized protein family
H14N18.1	H14N18.1: unc-23: Highly similar to mammalian BAG-2, BCL-2-associated aphanogene 2, a chaperone regulator
C40H1.5	C40H1.5: Member of an uncharacterized protein family
T23B3.2	T23B3.2: Protein of unknown function, has weak similarity to <i>C. elegans</i> F47B7.1
C25E10.8	C25E10.8: Member of an uncharacterized protein family
Y105C5A.12	Y105C5A.12: Protein of unknown function
B0507.8	B0507.8: Protein of unknown function, has similarity to <i>C. elegans</i> B0507.7
C25E10.9	C25E10.9: Member of an uncharacterized protein family
F38E11.1	F38E11.1: Member of the small heat shock protein family
Y51A2D.11	Y51A2D.11: Member of an uncharacterized protein family
Y51A2B.1	Y51A2B.1: Protein of unknown function, has moderate similarity to <i>C. elegans</i>

elegans C07G3.2
 C08E8.4: Protein of unknown function, has weak similarity to *C. elegans* C07G3.2
 C44H9.5: Protein of unknown function
 K10E9.1: Protein of unknown function
 C08F11.3: Protein of unknown function, has moderate similarity to *C. elegans* F56G4.1
 C01H6.6: Protein of unknown function, has weak similarity to *S. cerevisiae* Y01060p
 C24A11.2 C24A11.2
 W06D12.3: W06D12.3: fat-5: Likely a palmitoyl-CoA delta-9 fatty acid desaturase, specific for medium chain (14)
 T02B5.1: T02B5.1: Member of the carboxylesterase protein family
 C05E4.9: Putative ortholog of *S. cerevisiae* MLS1 gene product (Malate synthase 1, functions in glyoxylate cycle, has near identity to Dal7p)
 ZC395.5: ZC395.5: Protein of unknown function
 H24O09.c H24O09.c
 C24B9.9: C24B9.9: Protein of unknown function, has moderate similarity to *C. elegans* T04C12.1
 T20G5.7: T20G5.7: Protein of unknown function, has moderate similarity to *C. elegans* T20G5.8
 K07C6.4: K07C6.4: Member of the P450 heme-thiolate protein family
 B0213.15: B0213.15: Member of the P450 heme-thiolate protein family
 F09F7.6: F09F7.6: Protein of unknown function
 R03E9.1: R03E9.1: mtl-1: Member of the MAD family of putative transcription factors, interacts with *C. elegans* MAX-1
 B0238.1: B0238.1: Member of the carboxylesterase protein family
 E01G4.3: E01G4.3: Protein of unknown function
 M02D8.4: M02D8.4: Member of the asparagine synthetase protein family
 Y6E2A.3: Y6E2A.3: Protein of unknown function, has weak similarity to *C. elegans* Y6E2A.5
 F38E11.2: F38E11.2: hsp-12.6: Member of the small heat shock protein family
 F17B5.1: F17B5.1: Protein with strong similarity to *C. elegans* T20D4.7 gene product [Member of the thioredoxin protein family]
 K07A1.7: K07A1.7: Protein with similarity to *D. melanogaster* HDC (headcase) protein, a branching inhibitor produced by specialized tracheal cells
 F48D6.4: F48D6.4: Protein of unknown function
 F11A5.12: F11A5.12: Protein with similarity to estradiol 17-beta-dehydrogenases; strongly related to *C. elegans* C06B3.4, C06B3.5, F25G6.5, and C56G2.6
 C06B3.4: C06B3.4: Possible estradiol 17 beta-dehydrogenase, member of a protein family
 H10D18.2: H10D18.2: Putative paralog of *C. elegans* H10D18.4
 Y40B10A.6: Y40B10A.e Y40B10A.e
 ZK355.3: ZK355.3: Protein of unknown function
 K11G9.6: K11G9.6: mtl-1: Metallothionein-related, cadmium-binding intestinal protein
 C02A12.4: C02A12.4: Member of an uncharacterized protein family with weak similarity to *Entemeba histolytica* N-acetylmuraminidase
 AC3.7: AC3.7: Member of the UDP-glucuronosyltransferase protein family
 C54D10.3: C54D10.3: Member of an uncharacterized protein family

Upregulated in daf-2 mutants

<u>Gene</u>	<u>Gene product/homology</u>
ZK973.7	ZK973.7: Protein of unknown function, putative paralog of <i>C. elegans</i>
	ZK973_14.I
ZK973.7	ZK973.7: Protein of unknown function, putative paralog of <i>C. elegans</i>
	ZK973_14.I
ZK973.7	ZK973.7: Protein of unknown function, putative paralog of <i>C. elegans</i>
	ZK973_14.I
W10G6.3	W10G6.3: ifa-2: Putative intermediate filament protein
ZK270.2a	ZK270.2 ZK270.2
F16H6.7	F16H6.7: Member of an uncharacterized protein family
F57H12.7	F57H12.7: Protein of unknown function, has weak similarity to <i>C. elegans</i>
	W06B11.1
F32A5.5	F32A5.5: Protein with strong similarity to human AQP9, an aquaporin; putative paralog of <i>C. elegans</i> C01G6.1 and Y69E1A.G
C17G1.4	C17G1.4: Protein with moderate similarity to <i>H. sapiens</i> MUC1 gene product, a transmembrane mucin
K12G11.4	K12G11.4: Member of the alcohol dehydrogenase protein family
K12G11.3	K12G11.3: Member of the alcohol dehydrogenase protein family
F21F3.3	F21F3.3: Protein with strong similarity to <i>S. cerevisiae</i> Ste14p, a farnesyl cysteine
T07D10.4	T07D10.4: Member of the C-type lectin protein family
H16D19.1	H16D19.1: Member of the C-type lectin protein family
K11D2.2	K11D2.2: Putative acid ceramidase, has strong similarity to human ASAII, acid ceramidase (N-acylsphingosine amidohydrolase)
C52E4.1	C52E4.1: gcp-1: Cysteine protease expressed in the intestine
W08D2.4	W08D2.4: fat-3: Protein with delta6-fatty acid-desaturase activity
F53F4.13	F53F4.13: Protein of unknown function, has weak similarity to <i>C. elegans</i>
	F20A1.10
C26C6.3	C26C6.3: Member of the zinc metalloprotease protein family, has strong similarity to <i>C. elegans</i> TOH-2, has similarity to <i>D. melanogaster</i> and human TGF-beta-like growth factors
K08F4.7	K08F4.7: gst-4: Member of the glutathione S-transferase protein family, has similarity to human and <i>D. melanogaster</i> glutathione S-transferases
VC5.3	VC5.3: Ladder protein
VC5.3	VC5.3: Ladder protein
E01A2.8	E01A2.8: Putative paralog of <i>C. elegans</i> E01A2.7
K10B3.8	K10B3.8: gpd-2: Glyceraldehyde-3-phosphate dehydrogenase
R09B5.6	R09B5.6: Member of the hydroxyacyl-CoA dehydrogenase protein family
C56A3.2	C56A3.2: Member of an uncharacterized protein family
H22K11.1	H22K11.1: asp-3: Probable aspartyl protease and an ortholog of human cathepsin D
C06G8.1	C06G8.1: Protein of unknown function, has strong similarity to <i>C. elegans</i>
	K02D7.5, has similarity to <i>D. melanogaster</i> SLV (saliva), a putative transmembrane protein

Downregulated in daf-2 mutants and with daf-2 RNAi, upregulated with daf-16 RNAi

<u>Gene</u>	<u>Gene product/homology</u>
M60.1	M60.1: Putative serine proteinase, has strong similarity to human placental

ZK6.10	serine proteinase, P11 ZK6.10: Protein of unknown function, putative paralog of <i>C. elegans</i>
ZK6.11	
C49C3.9	C49C3.9: Protein of unknown function
F55G11.7	F55G11.7: Member of an uncharacterized protein family
F55G11.8	F55G11.8: Member of an uncharacterized protein family
K10D11.1	K10D11.1: Member of an uncharacterized protein family
C32H11.4	C32H11.4: Member of an uncharacterized protein family
Y46C8_103.a	Y46C8_103.a Y46C8_103.a
F56D6.2	F56D6.2: Member of the phospholipase A2 receptor protein family
B0365.6	B0365.6: Member of the C-type lectin protein family
ZK1127.10	ZK1127.10: Member of the cystathionine gamma-lyase protein family
C04F12.3	C04F12.3: Protein with similarity to human B-cell CLL/lymphoma 3 (BCL3)
T05A12.3	T05A12.3: Protein of unknown function, has weak similarity to <i>C. elegans</i>
R07G3.3	
C17H12.8	C17H12.8: Member of an uncharacterized protein family
C17B7.1	C17B7.1: G protein-coupled receptor, member of a large subfamily that contains ODR-10 odorant response protein, no homolog found in human or <i>D. melanogaster</i>
F15E11.1	F15E11.1 F15E11.1
F15E11.12	F15E11.12 F15E11.12
F22A3.6	F22A3.6: Possible lysozyme, member of an uncharacterized protein family
F58F6.2	F58F6.2: Putative collagen, has similarity to human COL9A1, alpha-1 collagen, type IX
B0478.1	B0478.1: jnk-1: Neuronally expressed serine/threonine protein kinase of the MAP kinase subfamily
F59D8.2	F59D8.2: vit-4: Member of the vitellogenin protein family; expressed only in <i>C. elegans</i> intestinal cells
F59D8.1	F59D8.f F59D8.f
ZK757.1	ZK757.1: Protein of unknown function with similarity to a DHHC zinc finger domain, putative paralog of <i>C. elegans</i> B0546.5 protein
R11G1.3	R11G1.3: Member of the glutathione S-transferase protein family, has similarity to <i>H. sapiens</i> and <i>D. melanogaster</i> glutathione S-transferases
C04F6.1	C04F6.1: vit-5: 170 kDa yolk protein
F41A4.1	F41A4.1: Protein with weak similarity to <i>C. elegans</i> let-653 (Putative mucin-like protein required for development beyond the late L1 larval stage or early L2 larval stage)
C54G4.6	C54G4.6: Protein with similarity to human ASMTL protein, acetylserotonin N-methyltransferase-like protein
Y19D10A.9	F15E11.9 F15E11.9
Y19D10A.9	Y19D10A.j Y19D10A.j
Y19D10A.9	F56A4.j F56A4.j
K08D8.5	K08D8.5: Member of an uncharacterized protein family
C32H11.1	C32H11.1: Member of an uncharacterized protein family
T03E6.7	T03E6.7: Member of the thiol protease protein family
R09H10.5	R09H10.5: Member of the EGF-repeat protein family
T25C12.3	T25C12.3: Member of the EGF-repeat protein family, member of the C-type lectin family
K12H4.7	K12H4.7: Member of the carboxypeptidase protein family

F28H7.3	F28H7.3: Member of the lipase protein family
Y38H6C.5	Y38H6C.5: Putative Zinc finger, CCHC class protein
F57F4.3	F57F4.3: Protein of unknown function, putative paralog of <i>C. elegans</i>
F57F4.4	F57F4.4: Protein of unknown function, putative paralog of <i>C. elegans</i>
F57F4.3	F57F4.3
C12C8.2	C12C8.2: Member of the cystathione gamma-lyase protein family
W01A11.4	W01A11.4: Member of the galactoside-binding lectin protein family
C08F11.8	C08F11.8: Member of the UDP-glucuronosyltransferase protein family
Y3SH6C.1	Y3SH6C.1: Protein of unknown function, has weak similarity to <i>C. elegans</i>
M02H5.B	
K06A4.5	K06A4.5: Putative 3-hydroxyanthranilate 3,4-dioxygenase, has strong similarity to human Hs.108441 gene product (3-hydroxyanthranilic acid dioxygenase)
F23H11.7	F23H11.7: Protein of unknown function
F52G3.4	F52G3.4: Protein with weak similarity to <i>C. elegans</i> F33H12.6
F46E10.1	F46E10.1 F46E10.1
C39E9.1	C39E9.1: Member of the testis-specific protein TPX-1 like protein family
C25F6.3	C25F6.3: Member of the 4Fe-4S ferredoxins and related iron-sulfur cluster binding protein family
F52E1.5	F52E1.5: Protein of unknown function
F28B4.3	F28B4.3: Member of the EGF-repeat protein family
Y106G6H.10	Y106G6H.10: Protein of unknown function, putative paralog of <i>C. elegans</i>
Y106G6H.9	Y106G6H.9: Protein of unknown function, putative paralog of <i>C. elegans</i>
Y106G6H.10	Y106G6H.10
K06A4.1	K06A4.1: Putative zinc metalloprotease with similarity over the N-terminus to human and <i>D. melanogaster</i> TOLLOID-like proteins, has strong similarity over the middle region to <i>C. elegans</i> F56A4.K and Y19D10A.K
B0281.5	B0281.5: Putative potassium voltage-gated channel, Shaw-related subfamily
F13A7.9	F13A7.9: Protein with moderate similarity to <i>S. cerevisiae</i> Skp1p, a component of the kinetochore complex and a component of SCF (Skp1p-cullin-F-box) complexes that target proteins for ubiquitin-dependent degradation; also has strong similarity to several other <i>C. elegans</i> proteins
Y51H7BR.2	Y51H7BR.2: Protein of unknown function, putative paralog of <i>C. elegans</i>
Y51H7BR.1	Y51H7BR.1
Y38E10A.14	Y38E10A.n Y38E10A.n
Y46H3C_14.c	Y46H3C_14.c Y46H3C_14.c
Y9D1A.1	Y9D1A.1: Protein with moderate similarity to <i>C. elegans</i> Y9D1A.A
Y49E10.8	Y49E10.8: Protein of unknown function
F56G4.3	F56G4.3: Protein of unknown function, is identical to <i>C. elegans</i> PES-2
F56G4.2	F56G4.2: pes-2: Protein of unknown function, is identical to <i>C. elegans</i> F56G4.3
C52D10.9	C52D10.9: Protein with similarity to the SKP1 family of proteins, nearly identical to <i>C. elegans</i> C52D10.7
C52D10.7	C52D10.7: Protein with similarity to the SKP1 family of proteins, nearly identical to <i>C. elegans</i> C52D10.9
Y56A3A.15	Y56A3A.15: Member of an uncharacterized protein family

C31A11.5	C31A11.5: G protein-coupled receptor, member of unnamed subfamily with distant homology to SRG subfamily, no homolog found in human or <i>D. melanogaster</i>
F49E11.7	F49E11.7: Member of the protein phosphatase protein family
C32H11.10	C32H11.10: Member of an uncharacterized protein family
C32H11.9	C32H11.9: Member of an uncharacterized protein family
ZK6.11	ZK6.11: Protein of unknown function, putative paralog of <i>C. elegans</i>
B0554.6	B0554.6: Member of an uncharacterized protein family
F55G11.5	F55G11.5: Member of an uncharacterized protein family
C32H11.12	C32H11.12: Member of an uncharacterized protein family
F35E12.5	F35E12.5: Member of an uncharacterized protein family
C08F8.5	C08F8.5: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.3	Y62H9A.3: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.4	Y62H9A.4: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.6	Y62H9A.6: Protein of unknown function, has weak similarity to <i>C. elegans</i>
Y62H9A.5	Y62H9A.5: Protein of unknown function, has weak similarity to <i>C. elegans</i>
F49E12.2	F49E12.2: Member of the calpain protease protein family
ZK896.8	ZK896.8: gcy-18: Protein with a cytoplasmic receptor tyrosine kinase domain and a guanylate cyclase domain, has strong similarity to human natriuretic peptide receptor NPR1 and photoreceptor-specific membrane retina guanylyl cyclase RetGC-2
T24B8.5	T24B8.5: Protein of unknown function, has weak similarity to <i>C. elegans</i>
K02H11.2	K02H11.2: G protein-coupled receptor, member of a large subfamily that contains ODR-10 odorant response protein, no homolog found in human or <i>D. melanogaster</i>
Y22F5A.5	Y22F5A.5: Member of an uncharacterized protein family with weak similarity to <i>Entemeba histolytica</i> N-acetylmuramidase
C25B8.3	C25B8.3: cpr-6: Member of the Cathepsin B-like Cysteine Protease family
Y55B1AR.1	Y55B1AR.1: Protein with weak similarity to <i>C. elegans</i> W09H1.6 (Galactoside-binding lectin)
F49C12.7	F49C12.7: Member of an uncharacterized protein family
ZK896.5	ZK896.5: Member of an uncharacterized protein family
W02D9.7	W02D9.7: Protein of unknown function
F49F1.1	F49F1.1: Member of an uncharacterized protein family

SEQUENCE LISTING

SEQ ID NO:1 Cytochrome C1 C54G4.8, Accession No. CAA99820.1

5 MQRAVVQGSK RGLAALAGVT AASGMGLVYA LENSVSASGD NVHPYALPWA
 HSGPFSSFDI ASVRRGYEVY KQVCAACHSM KFLHYRFVD TIMTEEEAKA
 EAADALINDV DDKGASIQRP GMLTDKLNP YPNKKAAAAA NNGAAPPDLS
 LMALARHGGD DYVFSLLTGY LEAPAGVKVD DGKAYNPYFP GGIISMPQQL
 FDEGIEYKDG TPATMSQQAK DVSAFMHWAA EPFHDTRKKW ALKIAALIPF
 10 VAVVLIYGKR HIWSFTKSQK FLFKTVKGRE PPKAQ

SEQ ID NO:2 NADH oxidoreductase T10E9.7, Accession No. AAB52474.1

MDSEILNYPPI HRFLDSSL LG QFIGPEGNSNI YKIEKYNKVA LDIWKNDEEN
 15 SNVRITGPYW NLKSALNDVL ELVSTIRKNK QRYKFEMPSK DIGFLIGKNG
 AKINEIKLSS NVDVHFERNN ENRDNGETDG RVKMLGSVIR QAVSRQIVRN
 SPISTTAAVA QTNQTGDKKE SPKKPTIWKI DEHKRERLAN FGKYAAECLP
 KFVQKVQFAA GDELELLIHP SGVVPVLSFL KGNHSAQFTN LTFITGMDVP
 TRKNRLEVIY SLYSVRFNAR VRVRTYTDEI APIDSATPVF KGADWFEREV
 20 YDMYGVWFNN HPDLRRILTD YGFEGHPFRK DYPLSGYNEV RYDPELKRVV
 YEPSELAQEF RKFDLNTPWE TFPAFRNQSI TSGYETILEV AEPTPATPQN K

SEQ ID NO:3 ATP synthase F27C1.7, Accession No. AAB37654.1

25 MAQLMKRGFS TSAALAKAQL VKTPIQVHGV EGRYAAALYS AGHKQNKL DQ
 ISTDLN NVRS VYKDNNKKFQE FVLDPTLKAN KKKTAIEAIS TKLGLTKETG
 NFLGLLAENG RLNKLESVVS SFESIMRAHR GELFVQVTS A EELSSSNQKA
 LSDALKIGK SGQKLT VTYA VKPSILGGLV VTIGDKYVDL SIASRVKKYK DALATAI

30 SEQ ID NO:4 cytochrome C oxidase F26E4.9, Accession No. CAB03002.1

MAQLAKTAVA ALSKKLVAPA AVARRT LATE ASPEDYGYYP DPLEHATGRE
 KKMLLARLAG DDRYE PKVYY RAEAST KQKP NLVPSHYDFR IIGCMCEQDS
 GHVNFM TIRK GDPKRCECGH WFKGV DADPE SI

SEQ ID NO:5 phosphoglucose isomerase Y87G2A.8, Accession No.

CAB60430.1

5 MSLSQDATFV ELKRHVEANE KDAQLLELFE KDPARFEKFT RLFATPDGDF
 LFDFSKNRIT DESFQLLMRL AKSRGVEESR NAMFSAEKIN FTENRAVLHV
 ALRNRAANRPI LVDGKDVMMPD VNRVLAHMKE FCNEIISGSW TGYTGKKITD
 VVNIGIGGSD LGPLMVTESL KNYQIGPNVH FVSNDGTHV AEVTKKLNAE
 TTLFIIASKT FTTQETITNA ETAKEWFLAK AGDAGAVAKH FVALSTNVTK
 10 AVEFGIDEKN MFEFWDWVGG RYSLWSAIGL SIAVHIGFDN YEKLLDGAFS
 VDEHFVNTPN EKNIPVILAM IGVLYNNIYG AETHALLPYD QYMHRFAAWF
 QQGDMESNGK FVTRHGQRVD YSTGPIVWGE PGTNGQHAFY QLIHQGTRLI
 PADFIAPVKT LNPIRGGLHH QILLANFLAQ TEALMKGKTA AVAEAEKLSS
 GMSPESIAKI LPHKVFEGNK PTTSIVLPVV TPFTLGALIA FYEHKIFVQG
 15 IIWDICSYDQ WGVELGKQLA KVIQPELASA DTVTSHDAST NGLIAFIKNN A

SEQ ID NO:6 GTPase gene T23H2.5, Accession No. AAC48200.1

MARRPYDMLF KLLIGDSGV GKTCILYRFS DDAFNTTFIS TIGIDFKIKT
 20 IELKGKKIKL QIWDTAGQER FHTITTSYYR GAMGIMLVYD ITNAKSFDNI
 AKWLRNIDEH ASEDVVKMIL GNKCDMSDRR VVSERERGEKI AQDHGISFHE
 TSAKLNVHVD TAFYDLAEAI LAKMPDSTDE QRSDTVNPVQ PQRQSSSGGC C

SEQ ID NO:7 product of LLW-1 gene Y54G11A.8, Accession No.

25 CAA22452

MLPSMSKLCT STVRPVAAAF STGTTTRQHHS SGRSRQQHHR HGGSGGKTNG
 GGRWSRYGKS AATGGTTVLA LSWMTTIKDV LGIEKVQLDA DPLKEKVQKS
 WLYRKRRQYD DAIQVLQLAL EEAERKEDM PITRVYDEMA NTFYEKMNL
 30 EADKYFRIVI QRLVQLHGKK DFDPEFIGVS LKLADILAH R GDLESAESGF
 KHCVRQMKV MEEHMKKFSV AHGALVEDRH TVDTFGPMYT DPIALFGMTL
 EAYANFLINY CGETRMAEVE EYIDEVMKIS YQIYGASSAH TINMLNNFGA
 TLVLKNRFEL AKYLAIGVD RILYVNECAH MLPGYYCNYA ESLFHTGQKN
 EALEFARKAV QMSRSGDDRV RHYTQNFLND LEKDINRGKP KSWWFF

SEQ ID NO:8 product of LLW-2 gene F59E12.10, Accession No. AAB54251

MNASSRTKPA IDLNKVPPID HHRTAVTFNC LIMKMTEMLN NFGNKMEDIL
 5 EKAEQSLDTA DRKLRLMESK LAGMSLEDKS TTATPSSAPE IDEIHESNPS
 SSQIVEETVE EKPEEHTTV LIKDDPAYSK YFKMLKLGVL EAGVIQKMK
 EGVDPSILKR GDEPSRPQAQ TSRNYESSGE STASFSDSD

SEQ ID NO:9 LLW-3 gene Y48E1B.1, Accession No. CAB07688

10 MYHVPLIPRD AGREETIFRI NQSLQKLLRV SDEIFDRVEH RITRIHGKAE
 AIDRRTEVLE KKLESIQESD KVITFTLPRQ LPKLPEEPPT STSLFRINID
 TEHFPGSEEL PAFRRADDHV LRPCEPIDFT YELNKPDKFF LTSQVLKEYE
 QKGWERYKKR LLGGLRELSR SPEHIAELFY AGTSIPAFEG VSGDFSKKAL
 15 DADDDGGTSR SGRTTDELAQ LRLHEQLL ED TALSSTLMQE DSLDDNHPLA
 FRINFNEKKK KTAKMVEMPD SLPNLKGHAH DFTLRDPEID EDRLLDILPA
 DDQIPEASEP TEAEADAPTT FILPPPPPM KLDPSQPAA TPVEITEIPP
 IISPPAPPBP PPPPPPPP QTPSASSSVT FSPTKSVDGG RSDLMAAIRA
 AGGAGNAKLS RIAEKPKRKG KFDGILESSA LLGASETPRN SAPAPDG
 20 GGDLMSALSK ALDARRKAIN GKVEAQPPAK VSSTIPAPPN FDDEEW
 D

SEQ ID NO:10 product of LLW-4 gene F45H10.4 , Accession No. CAB04386

25 MIIACSLIASLIPTVIGLKSNRSCFHYSCLATEANLKQCAGGTAISLTLEAKDVNIR
 DLVKYRALEFVGQCDRLLKEVVDFESLQILVNEDARECFEKLPESSKRVEEFTDSCDYVQPV
 SRNASKGDALQCLIEFKQDREYCESLLECCPDHTRCGERMNAVLSYQARVKAEQIVYSMI
 SCIVVNDPRFLREGARLQLSPRLPYRNAGVPFLRPDAYTEARITRRLALTSTATLSQRERFL
 KKYSQIRQVVAQNLFGQQQLTRPVEDLVTETSSKLAVEEAPEETTQEETTDASEVTTK
 AVEEATEEVTEEATEATEAPVATTKESSEMHVNTIRNMIRSASEKDLSKYVTLISEGKFSEL
 30 FELAEQKLTLSKFDKLSSKMAKLKDLINEALSEKEKSGIEQAMEKFEKPEKSELVAME
 DKDTPAVFTISDSLKHKKAEAKLAHTIVSRNVVEAENAIEKEVVEPKAEKKVKEEDVKAVA
 EEKKEEKPGKLPMKIEKLEKPVDTKSENHELKKVLDKERALLVESEIKNTAEETKPKVES
 FKSEETTVIAIDMPALEKEESAEEKKETTGEPTTTEAAVETTEASETPKPEAKPELNSLEDV
 LTLLTPETETIEGSGEREETTSAPAAEATSEITLLKSSSDVAVIENVKRIRPRTEQTHCQO
 35 YASCWQTVLVDYEQQCDRKYSTEVLSHGIDDSEIILNILHNSSISHHEIVLKACLRPLDRSVHS
 TLKQLLVIQRGVRKACLELGRNKIAVTDSEEALCNEIPSTADEFISSEHVRSQSNHLTC
 RAKLEPIRETCISVRNCCASVDTCDNYISSSPVKKLETEAIRRLVKKONDSETKMLQTLSYI
 HEQLSNPSRRRFYYH

SEQ ID NO:11 product of HSF-1 gene Y53C10A.12, Accession No.

CAA22146

MQPTGNQIQQ NQQQQQQQLIM RVPKQEVSVS GAARRYVQQA PPNRPPRQNH
5 QNGAIGGKKS SVTIQEVPNN AYLETLNKSG NNKVDDDKLP VFLIKLWNIV
EDPNLQSIVH WDDSGASFHI SDPYLFGRNV LPHFFKHNNM NSMVRQLNMY
GFRKMTPLSQ GGLTRTESDQ DHLEFSHPCF VQGRPELLSQ IKRKQSARTV
EDKQVNEQTQ QNLEVVMADM RAMREKAKNM EDKMNKLTK E NRDMWTQMGS
MRQQHARQQQ YFKKLLHFLV SVMQPGLSKR VAKRGVLEID FCAANGTAGP
10 NSKRARMNSE EGPyKDVC DL LESLQRETQE PFSRRFTNNE GPLISEVTDE
FGNSPVGRGS AQDLFGDTFG AQSSRYSDGG ATSSREQSPH PIISQPQSNS
AGAHGANEQK PDDMYMGSGP LTHENIHRCGI SALKRDYQGA SPASGGPSTS
SSAPSGAGAG ARMAQKRAAP YKNATRQMAQ PQQDYSGGFV NNYSGFMPSD
PSMIPIYQPSH QYLQPHQKLM AIEDQHHPTT STSSTNADPH QONLYSPTLGL
15 SPSFDRQLSQ ELQEYFTGTD TSLESFRDLV SNHNWDDFGN NVPLDDDEEG
SEDPLRQLAL ENAPETSNYD GAEDLLFDNE QQYPENGFDV PDPNYLPLAD
EEIFPHSPAL RTPSPSDPNL V

WHAT IS CLAIMED IS:

1. A method for identifying a compound that modulates aging, the
2. method comprising the steps of:
 3. (i) contacting the compound with a polypeptide, wherein the polypeptide is
4. encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5. encoding a polypeptide comprising an amino acid sequence selected from the group
6. consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2),
7. ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose
8. isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2
9. (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), LLW-4 (SEQ ID NO:10) and HSF-1 (SEQ ID
10. NO:11), and human homologues thereof; and
 11. (ii) determining the functional effect of the compound upon the
12. polypeptide.
1. 2. The method of claim 1, wherein the polypeptide is encoded by a
2. nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a
3. polypeptide comprising an amino acid sequence selected from the group consisting of:
4. cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase
5. (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ
6. ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8),
7. LLW-3 (SEQ ID NO:9), and LLW-4, (SEQ ID NO:10) or human homologues thereof.
1. 3. The method of claim 1, wherein the polypeptide is encoded by a
2. nucleic acid that hybridizes under stringent conditions to a nucleic acid encoding a
3. polypeptide comprising an amino acid sequence of HSF-1 (SEQ ID NO:11), or human
4. homologues thereof.
1. 4. The method of claim 1, wherein the functional effect is determined
2. *in vitro*.
1. 5. The method of claim 4, wherein the functional effect is determined
2. by measuring enzymatic activity.
1. 6. The method of claim 4, wherein the functional effect is determined
2. by measuring ligand, substrate, or cofactor binding to the polypeptide.

1 7. The method of claim 4, wherein the functional effect is determined
2 by measuring binding to a promoter sequence by the polypeptide.

1 8. The method of claim 1, wherein the polypeptide is expressed in a
2 mitochondrial extract or an isolated mitochondrial membrane.

1 9. The method of claim 1, wherein the polypeptide is expressed in a
2 eukaryotic host or host cell and the polypeptide is contacted with the compound in a
3 living cell.

1 10. The method of claim 9, wherein the host cell is derived from *C.*
2 *elegans*, mouse, rat, or human.

1 11. The method of claim 9, wherein the host is *C. elegans*, mouse, rat,
2 or human.

1 12. The method of claim 9, wherein the functional effect is a
2 determined by measuring ligand, substrate, or cofactor binding to the polypeptide.

1 13. The method of claim 9, wherein the functional effect is determined
2 by measuring transcriptional activation.

1 14. The method of claim 9, wherein the functional effect is determined
2 by evaluating age-associated parameters.

1 15. The method of claim 9, wherein the functional effect is determined
2 by evaluating expression of an age-associated gene.

1 16. The method of claim 14, wherein the age-associated parameter is
2 lifespan.

1 17. The method of claim 1, wherein the modulation is inhibition of
2 aging.

1 18. The method of claim 17, wherein inhibition of aging occurs by
2 activation of an HSF polypeptide encoded by a nucleic acid that hybridizes under
3 stringent conditions to a nucleic acid encoding a polypeptide comprising an amino acid
4 sequence of HSF-1 (SEQ ID NO:11), or human homologues thereof.

1 19. The method of claim 17, wherein inhibition of aging occurs by
2 inhibition of a polypeptide encoded by a nucleic acid that hybridizes under stringent
3 conditions to a nucleic acid encoding a polypeptide comprising an amino acid sequence
4 selected from the group consisting of: cytochrome C₁ (SEQ ID NO:1), NADH
5 oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3), cytochrome C oxidase
6 (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6),
7 LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), and LLW-4
8 (SEQ ID NO:10) or human homologues thereof

1 20. The method of claim 1 wherein the polypeptide is recombinant.

1 21. The method of claim 1, wherein the compound is an antibody, an
2 antisense molecule, or a small molecule.

1 22. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide, wherein the polypeptide is
4 encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5 encoding a polypeptide comprising an amino acid sequence selected from the group
6 consisting of: cytochrome C₁ (SEQ ID NO:1), NADH oxidoreductase (SEQ ID NO:2),
7 ATP synthase (SEQ ID NO:3), cytochrome C oxidase (SEQ ID NO:4), phosphoglucose
8 isomerase (SEQ ID NO:5), GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2
9 (SEQ ID NO:8), LLW-3 (SEQ ID NO:9), LLW-4 (SEQ ID NO:10) and HSF-1 (SEQ ID
10 NO:11), and human homologues thereof;

11 (ii) determining the functional effect of the compound upon the
12 polypeptide; and

13 (iii) contacting a host or host cell expressing the protein and evaluating an
14 age-associated parameter in a host or host cell, thereby identifying a compound that
15 modulates aging.

1 23. The method of claim 22, wherein the polypeptide is recombinant.

1 24. The method of claim 22, wherein the functional effect is a physical
2 effect.

1 25. The method of claim 22, wherein the functional effect is a chemical
2 effect.

1 26. The method of claim 22, wherein the functional effect is a
2 phenotypic effect.

1 27. The method of claim 22, wherein the functional effect is
2 determined *in vitro*.

1 28. The method of claim 22, wherein the functional effect is
2 determined in a eukaryotic host organism or host cell.

1 29. The method of claim 22, wherein the age-associated parameter is
2 lifespan.

1 30. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide that is a component of the
4 mitochondrial respiratory chain; and
5 (ii) determining the functional effect of the compound upon the
6 polypeptide.

1 31. The method of claim 30, wherein the polypeptide is expressed in a
2 mitochondrial extract or an isolated mitochondrial membrane.

1 32. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide, wherein the polypeptide is
4 encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5 listed in Table 5 or Table 6, or a nucleic acid encoding a polypeptide listed in Table 5 or
6 Table 6, or human homologues thereof; and
7 (ii) determining the functional effect of the compound upon the
8 polypeptide.

1 33. A method for identifying a compound that modulates aging, the
2 method comprising the steps of:

3 (i) contacting the compound with a polypeptide, wherein the polypeptide is
4 encoded by a nucleic acid that hybridizes under stringent conditions to a nucleic acid
5 listed in Table 5 or Table 6, or a nucleic acid encoding a polypeptide listed in Table 5 or
6 Table 6, or human homologues thereof

(ii) determining the functional effect of the compound upon the polypeptide; and

9 (iii) contacting a host or host cell expressing the protein and evaluating an
10 age-associated parameter in a host or host cell, thereby identifying a compound that
11 modulates aging.

1 34. A compound that modulates an aging process, wherein the
2 compound is identified by the method of claim 1.

1 35. The compound of claim 34, wherein the compound is an antibody,
2 an antisense molecule, or a small molecule.

1 36. The compound of claim 34, wherein the compound is antimycin or
2 an analog thereof.

1 37. A method of increasing lifespan or treating premature aging in a
2 subject, the method comprising the step of administering to the subject an effective
3 amount of a compound identified using the method of claim 1.

38. The method of claim 37, wherein the compound is antimycin.

1 39. A method of increasing lifespan or treating premature aging in a
2 subject, the method comprising the step of administering to the subject an effective
3 amount of a compound that modulates the expression of a polypeptide comprising an
4 amino acid sequence selected from the group consisting of: cytochrome C₁ (SEQ ID
5 NO:1), NADH oxidoreductase (SEQ ID NO:2), ATP synthase (SEQ ID NO:3),
6 cytochrome C oxidase (SEQ ID NO:4), phosphoglucose isomerase (SEQ ID NO:5),
7 GTPase (SEQ ID NO:6), LLW-1 (SEQ ID NO:7), LLW-2 (SEQ ID NO:8), LLW-3 (SEQ
8 ID NO:9), LLW-4 (SEQ ID NO:10) and HSF-1 (SEQ ID NO:11), and human
9 homologues thereof.

1 40. The method of claim 37 or 39, wherein the aging process is
2 abnormal.

1 41. The method of claim 40, wherein the abnormal aging process is
2 selected from Werner syndrome, Hutchinson-Guilford disease, Bloom's syndrome,
3 Cockayne's syndrome, ataxia telangiectasia, and Down's syndrome.

1 42. The method of claim 37 or 39, wherein the aging process is normal.

1 43. The method of claim 37 or 39, further comprising the step of
2 evaluating an age-associated parameter of the subject.

1 44. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:

3 (i) providing a library of nucleic acids, each nucleic acid of the library comprising
4 a segment of a gene of an organism; and

5 (ii) for each member of the library,

6 (a) generating double-stranded RNA (dsRNA) from the respective member
7 of the library,

8 (b) providing the dsRNA to a cell or one or more cells of the organism to
9 provide a ds-RNA treated cell or ds-RNA treated organism, and

10 (b) monitoring an age-associated parameter of the ds-RNA treated cell or
11 the ds-RNA treated organism.

1 45. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:

3 (i) evaluating a relationship between presence or abundance for each species of a
4 plurality of RNA or protein species with respect to age of a cell or organism;

5 (ii) producing or delivering a double-stranded RNA to a cell or one or more cells
6 of an organism, the RNA corresponding to a species whose presence or abundance is
7 correlated with age; and

8 (iii) monitoring an age-associated parameter of the cell or the one or more cells or
9 the organism.

1 46. A method of identifying a compound that modulates aging, the
2 method comprising the steps of:
3 (i) contacting a test compound to a living or biochemical system that comprising a
4 *C. elegans* target protein selected from the group consisting of: a respiratory chain
5 component, a heat shock promoter activator, LLW-1, LLW-2, LLW-3, LLW-4, a small
6 GTPase, a protein in Table 5, and a protein in Table 6; and
7 (ii) evaluating a property associated with the target protein; and
8 (iii) evaluating an aging-associated parameter of a *C. elegans* organism contacted
9 with the test compound.

1 47. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:
3 (i) providing a nematode in which activity of a target protein is reduced in the
4 organism by RNA interference;
5 (ii) expressing a gene encoding a candidate mammalian protein that is
6 heterologous to the organism; and
7 (iii) evaluating an age associated parameter of the organism.

1 48. A *C. elegans* nematode that (1) expresses a heterologous gene in at
2 least some cells, the heterologous gene encoding a respiratory chain component or
3 glycolysis component that is non-identical to the corresponding endogenous respiratory
4 chain component or glycolysis component, or a functional domain thereof; and (2) is
5 deficient in at least some cells for an endogenous activity provided by the corresponding
6 endogenous respiratory chain component or glycolysis component.

1 49. A method comprising: assessing an age-associated parameter of the
2 nematode of claim 48.

1 50. A *C. elegans* nematode that (1) has a deficiency in at least some
2 cells for an endogenous activity, the deficiency generated by dsRNA in the cells, and (2)
3 has an average lifespan of at least 40% greater than an otherwise identical nematode
4 without the deficiency.

1 51. A method of identifying a gene or gene product that modulates
2 aging, the method comprising the steps of:

3 (i) providing the nematode of claim 50;

4 (ii) introducing a heterologous gene that encodes a heterologous polypeptide into

5 the nematode;

6 (iii) expressing the heterologous gene in the nematode or a progeny of the

7 nematode under conditions wherein the heterologous polypeptide is produced; and

8 (iv) monitoring an age-associated parameter of the nematode or the progeny of the

9 nematode.

1 52. The method of claim 51, further comprising contacting a test

2 compound to the nematode or the progeny prior to or during the monitoring.

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RNAi of respiratory chain and glycolysis
genes increases life span

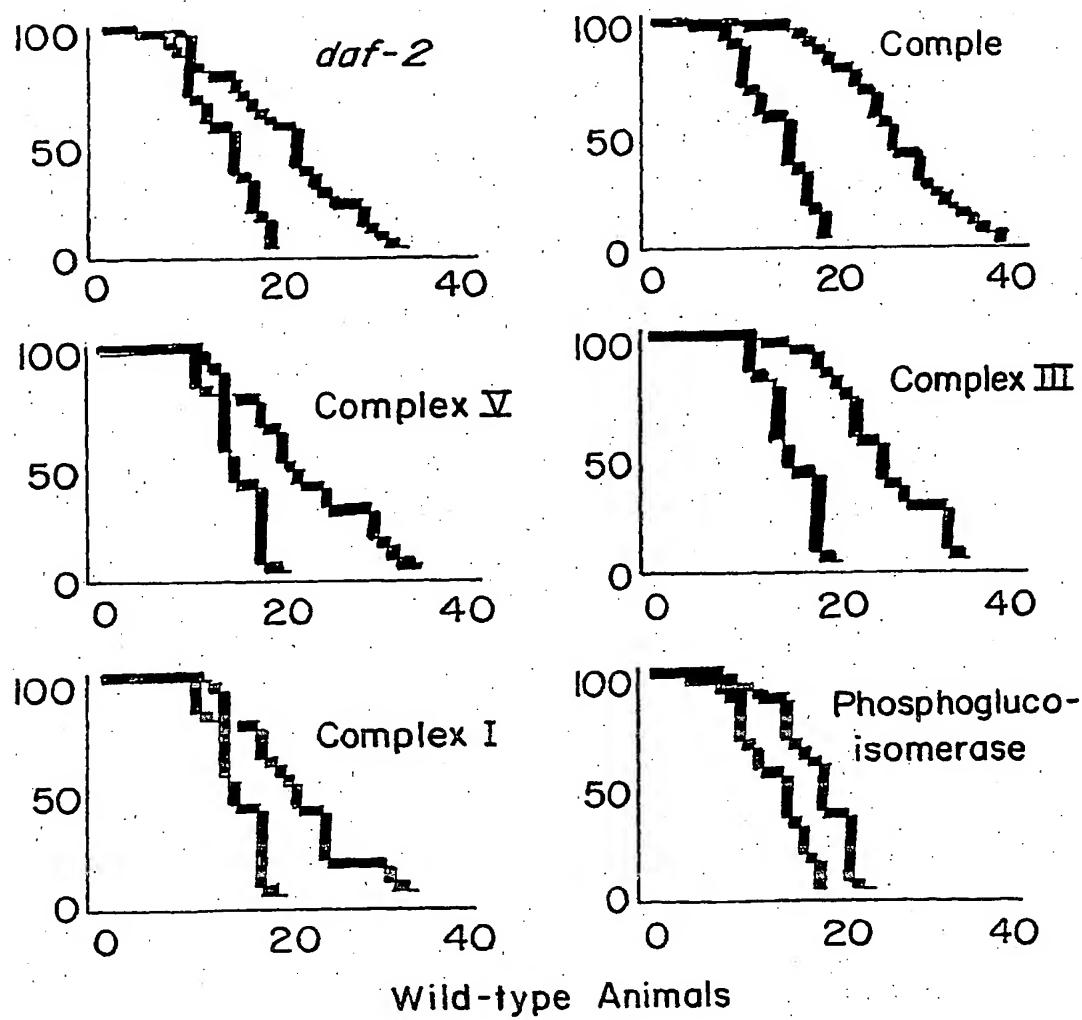
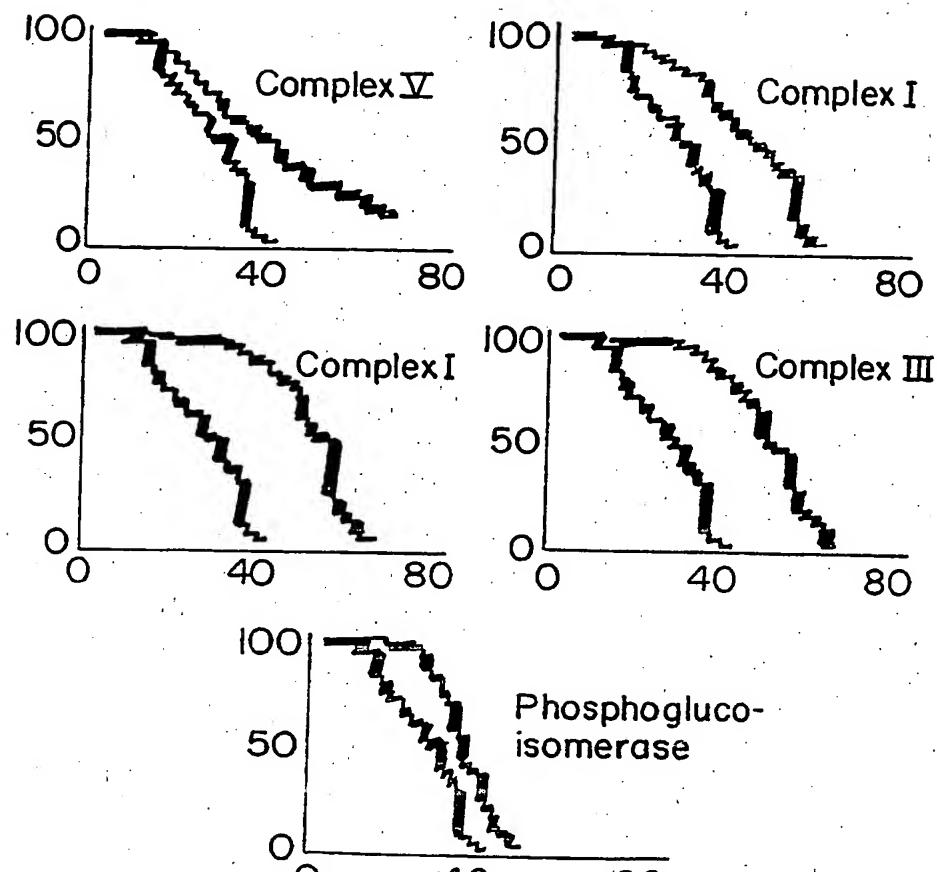


FIG. 1.

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daf-2 (e1370) worms treated with dsRNAs
of respiratory and glycolytic genes



daf-2 (e1370) Animals

FIG. 2.

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Antimycin increases life span
of *C. elegans*

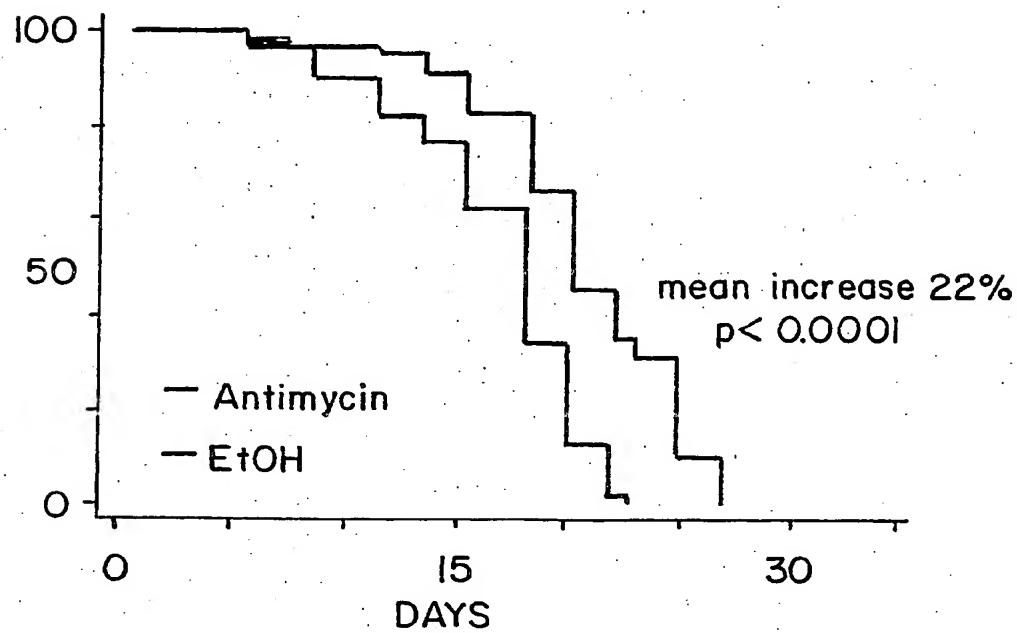
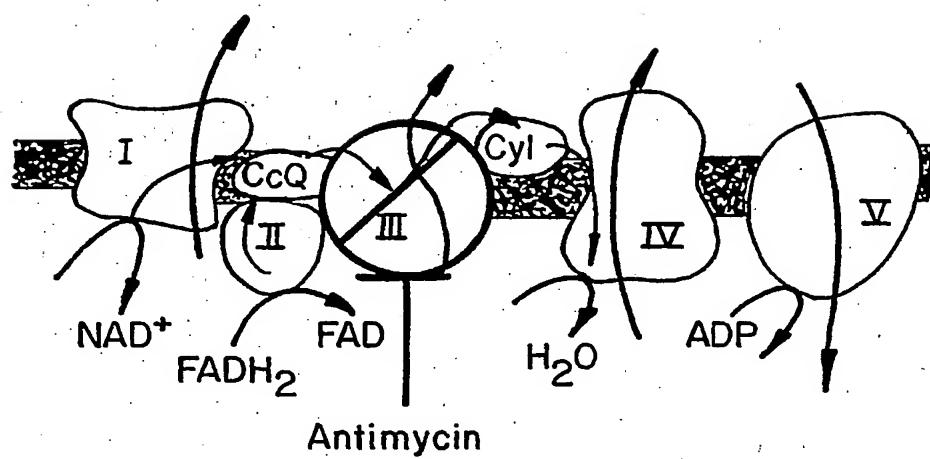


FIG. 3.

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daf-16(mu86) mutant worms
on different dsRNA

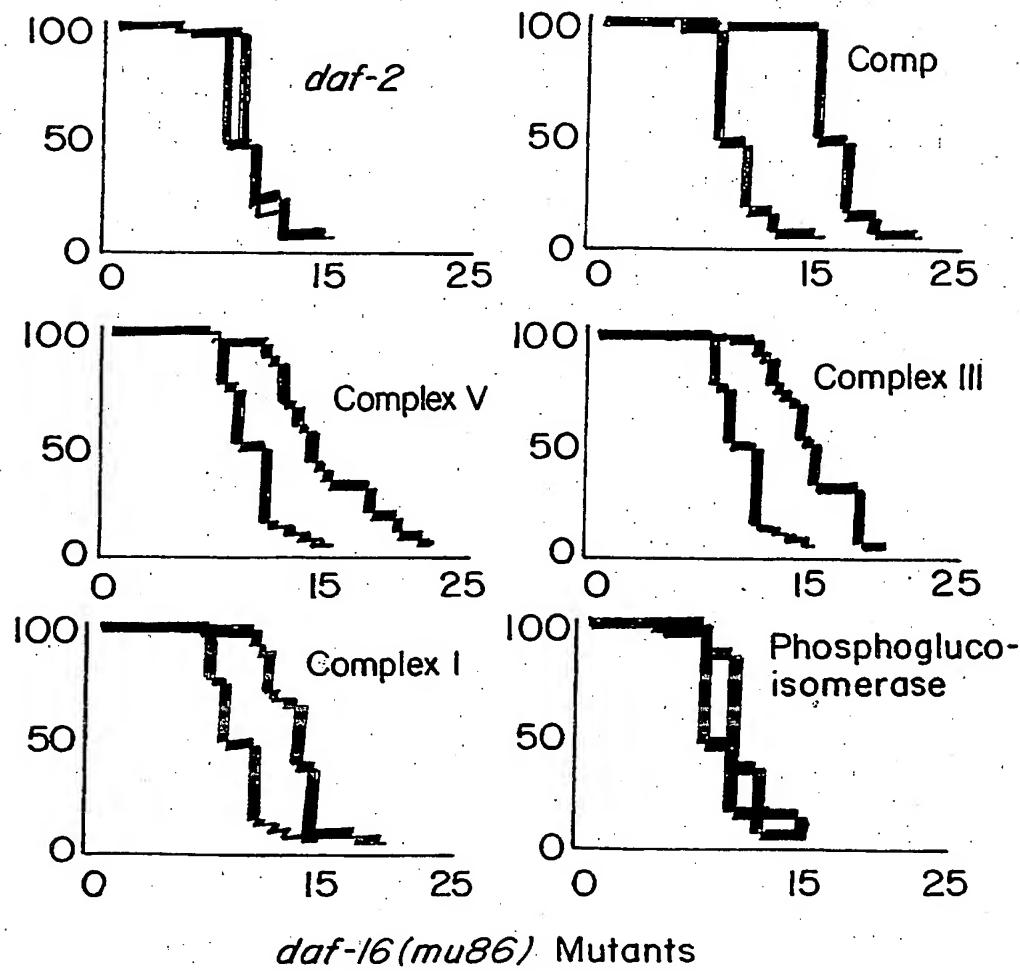


FIG. 4.

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Group 1: Genes that are upregulated in daf-2(-) wormsOxidative stress protection

sod-3: Manganese superoxide dismutase

ctl-1: cytosolic catalase

ctl-2: Peroxisomal catalase

- ④ mtl-1: Metallothionein; cadmium-binding glutathione S-transferase protein family-2
- ④ hsp-12.6, sip-1, hsp-16.1, hsp-16.11

Metabolic

acyl-CoA synthetase

alcohol dehydrogenase

Malate synthase 1/isocitrate lyase

acyl CoA dehydrogenase

hydroxyacyl-CoA dehydrogenase

dao-3: C1-tetrahydrofolate synthase

trehalase

trehalose-6-phosphate synthase

acyl-CoA oxidase

Steroid metabolism/lipid synthesis

CytP450 heme-thiolate protein family-3

UDP-glucuronosyltransferase protein family-4

estradiol 17 beta-dehydrogenase-2

fat-6: stearoyl-CoA delta-9 fatty acid desaturase

fat-7: stearoyl-CoA delta-9 fatty acid desaturase

fat-3: delta6-fatty acid-desaturase activity

ASA-H acid ceramidase

aminopeptidases--many

Carboxypeptidases--many

Antibacterial, antifungal

Thaumatin-like antifungal-3

Bactericidal amoebapores; antibacterial agent

Pathogen-related in yeast (PRY) protein family

N-acetyl muramidase-2

Many unknown genes/
members of unknown familiesProteolysis

ges-1: Carboxylesterase expressed in gut cells

gcp-1: Cysteine protease expressed in the intestine
cathepsin Z-like cysteine protease

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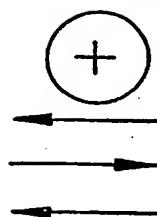
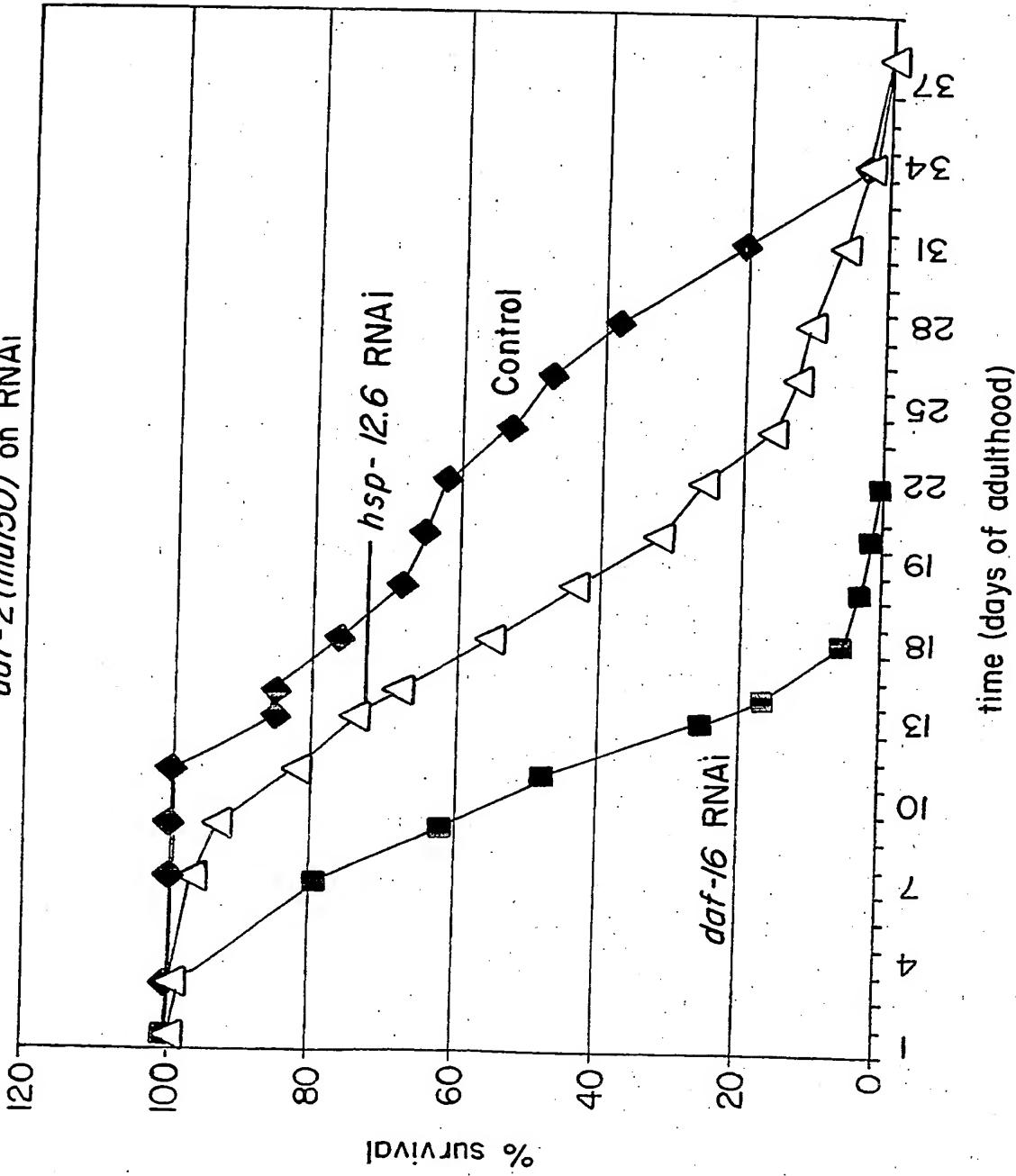
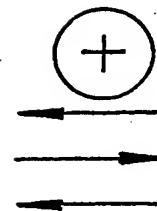
*hsp-12.6* RNAi reduces *daf-2* longevity*daf-2(mu50)* on RNAi

FIG. 6.

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...as does *mtl-1* RNAi
daf-2 (mu50) on RNAi

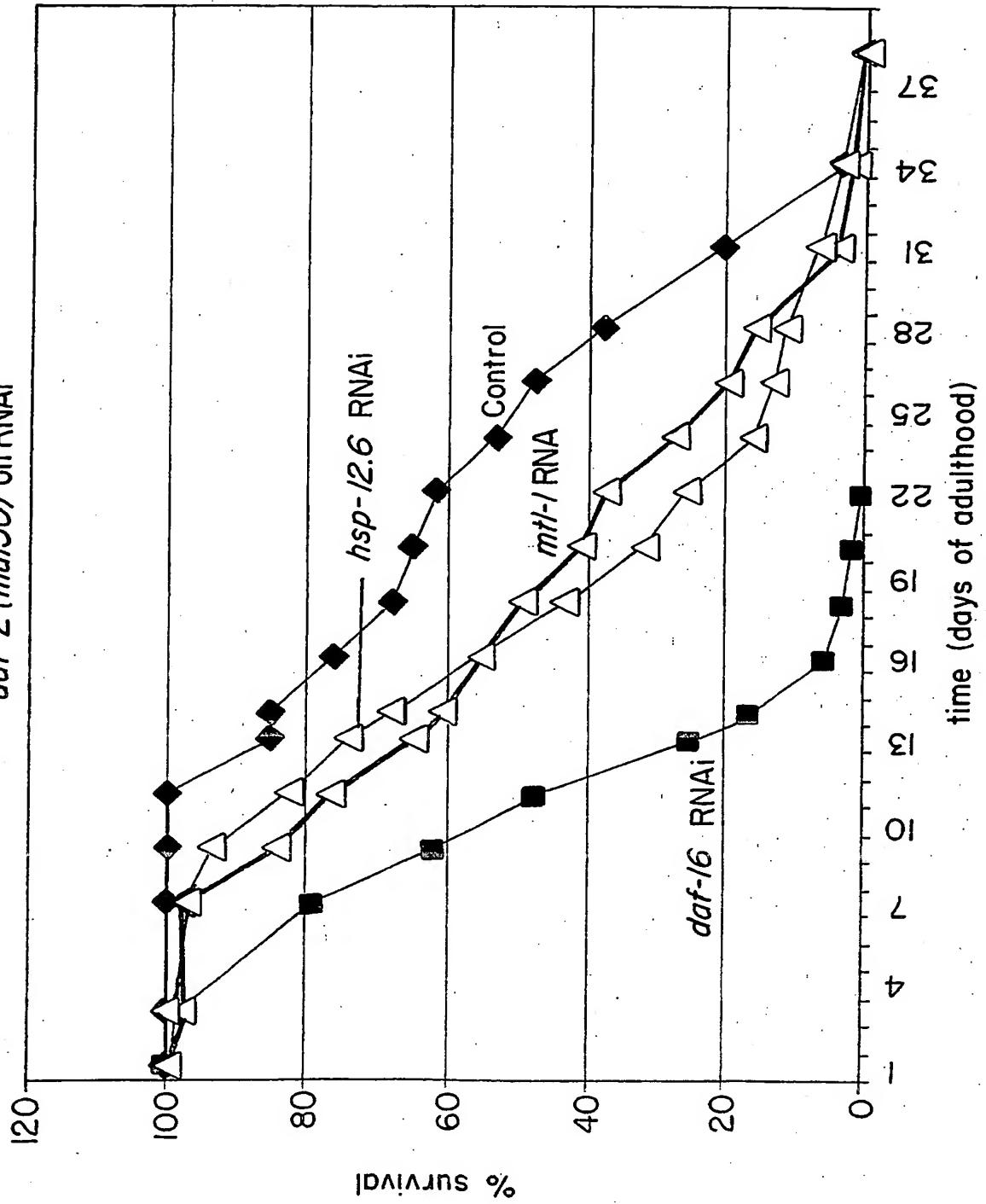


FIG. 7

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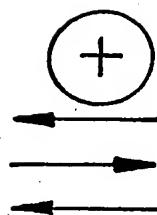
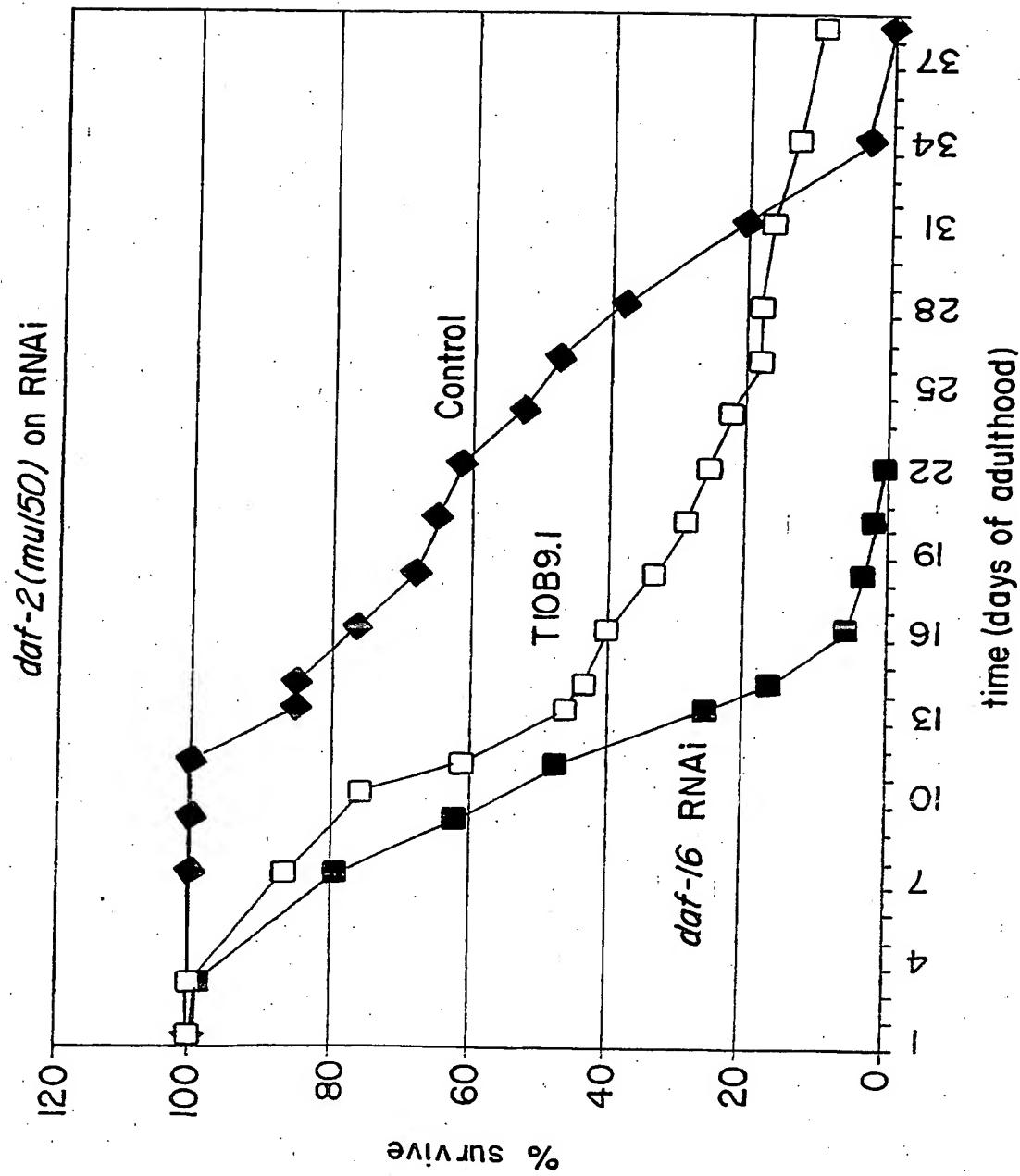
A cytochrome P450 shortens *daf-2* lifespan

FIG. 8.

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Group 2: Genes downregulated in daf-2 mutantsNeuronal

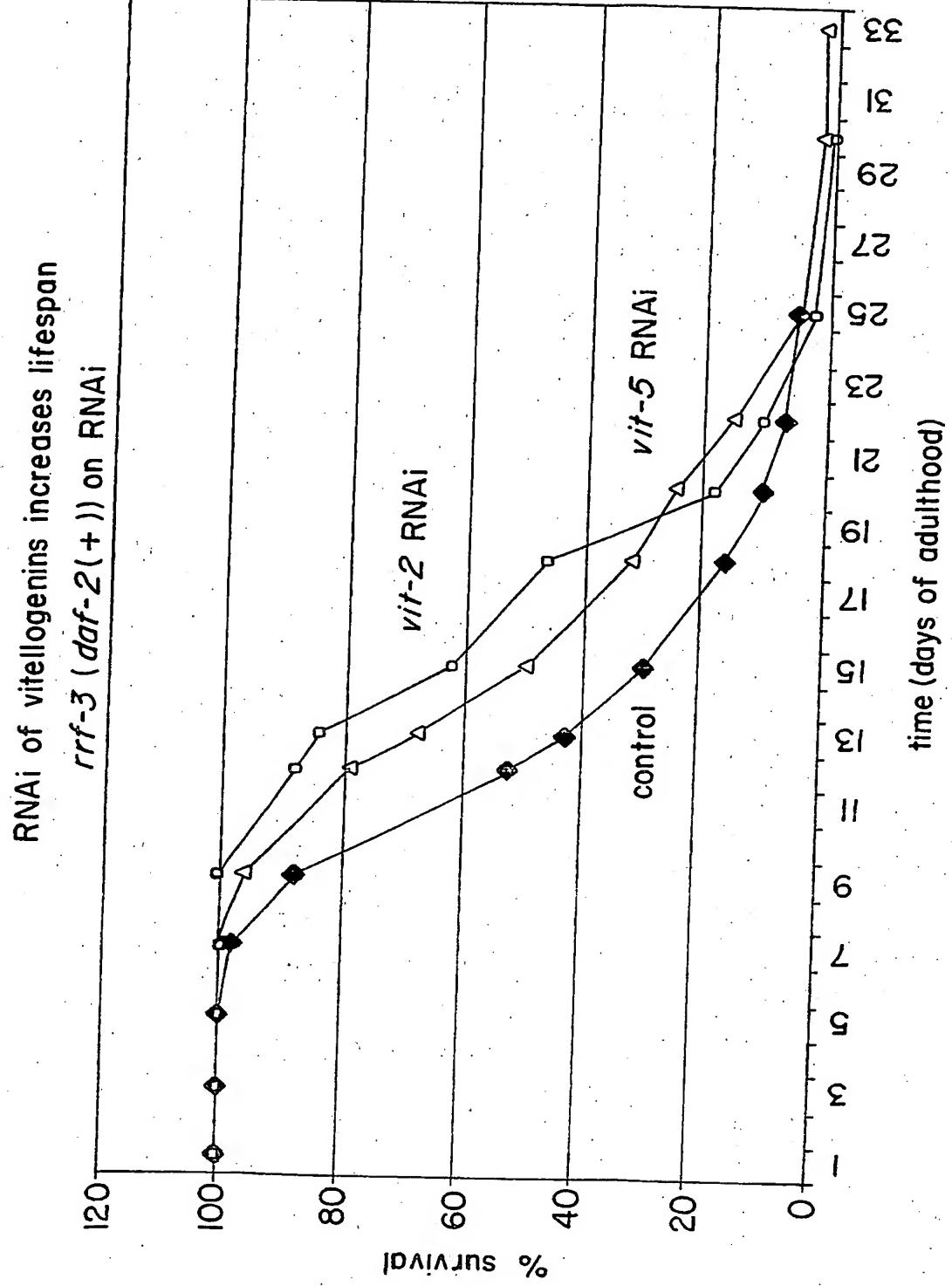
- similarity to human ASMTL protein, acetylserotonin N-methyltransferase-like protein
- Putative G protein-coupled receptor, no homolog found in human or D. melanogaster
- gcy-18: Protein with a cytoplasmic receptor tyrosine kinase domain and a guanylate cyclase domain,
- has strong similarity to human natriuretic peptide receptor NPR1
- gcy-6: Putative guanylyl cyclase expressed in the ASEI neuron

Other (most are unidentified)

- vitellogenins
- nhr-64: Member of the nuclear hormone receptor/Zinc finger protein family
- pep-2: Member of the proton-coupled oligopeptide transporter superfamily
- mtl-2: Metallothionein-related, cadmium-binding protein
- lbp-7: Member of the fatty acid-binding protein family
- aminopeptidases--many
- Carboxypeptidases--many

- ins-7: Insulin-related protein of the type-beta subfamily

FIG. 9.

*FIG. 10.*

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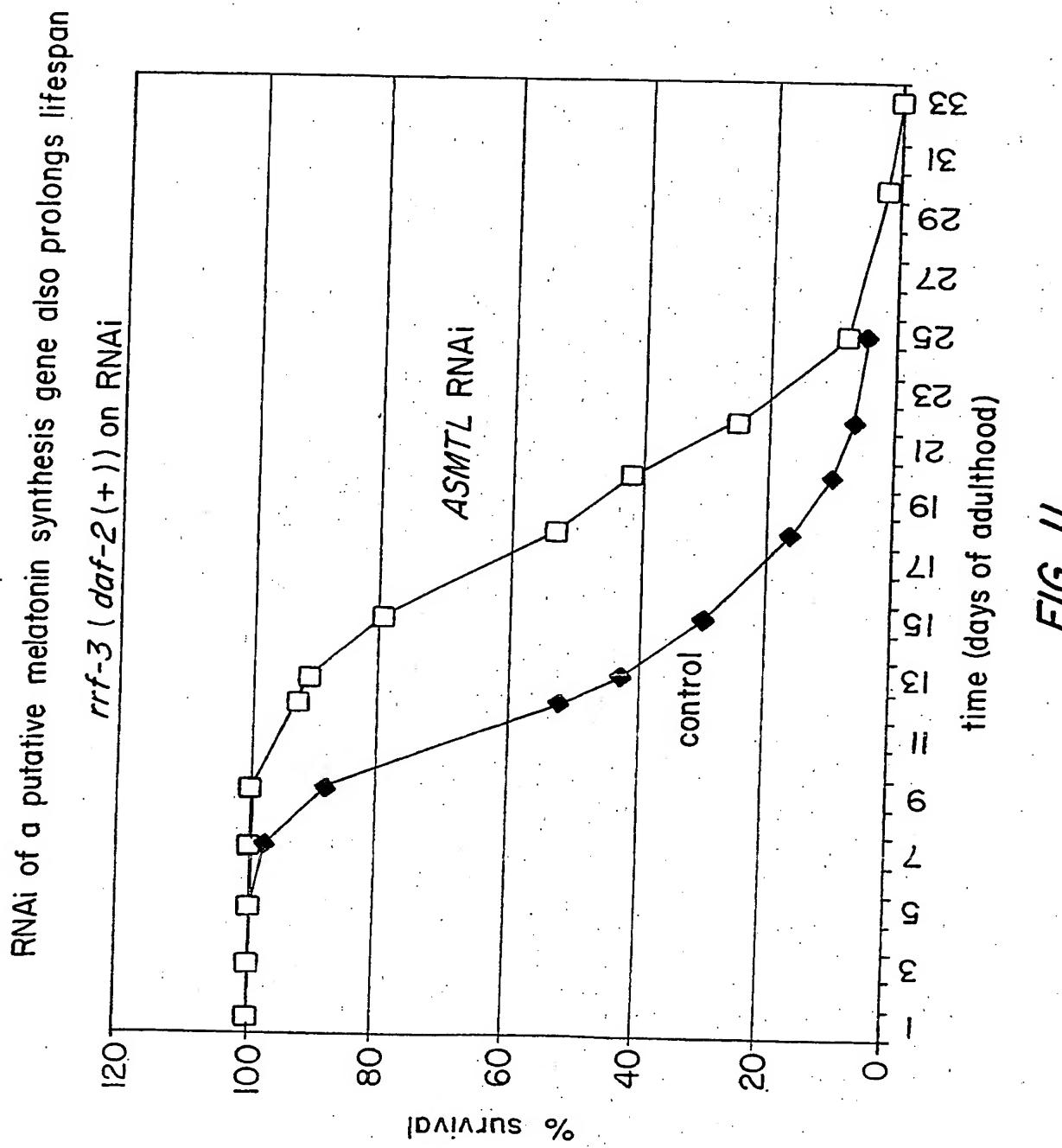


FIG. II.

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DAF-16 increases lifespan through a combination of induction and repression of lifespan-affecting genes

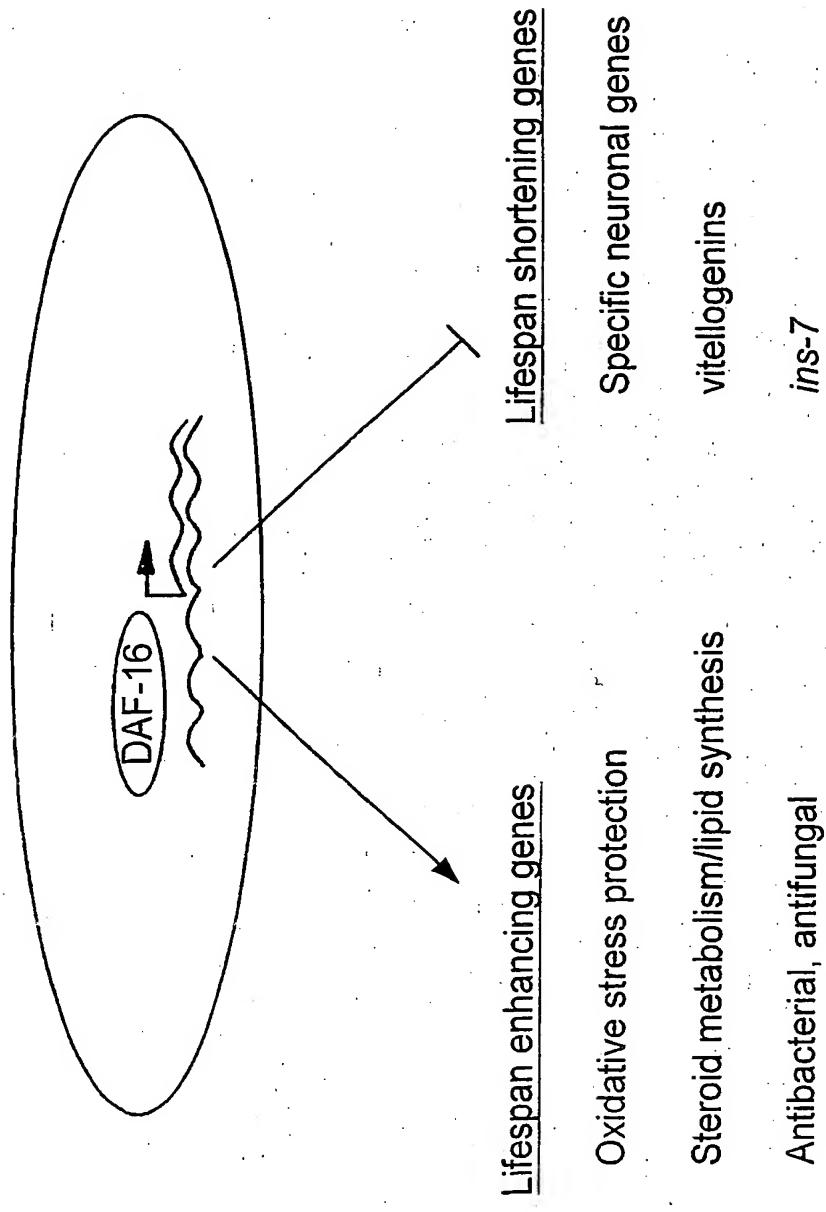


FIG. 12.

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Declaration under Rule 4.17:

— *of inventorship (Rule 4.17(iv)) for US only*

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For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: EUKARYOTIC GENES INVOLVED IN ADULT LIFESPAN REGULATION

WO 03/000861 A3

(57) Abstract: The present invention relates to regulation of adult lifespan in eukaryotes. More particularly, the present invention is directed to methods of assaying for genes, gene products, and genes in pathways controlled by such genes and gene products, using RNAi and microarray analysis, that regulate lifespan (e.g., extend or truncate adult lifespan) in eukaryotes such as invertebrates (e.g., C. elegans), plants, and mammals, e.g., humans. For example, the present invention is directed to genes encoding components of the mitochondrial respiratory chain and genes encoding glycolysis enzymes, which are involved in lifespan regulation, and genes and gene products in pathways controlled by such genes. Other genes and gene products identified as regulating aging and aging pathways include a gene encoding a GTPase; a transcriptional activator; novel genes: llw-1, llw-2, llw-3, and llw-4; genes encoding cytochrome P450 proteins (involved in steroid biosynthesis); a melatonin synthesis gene; genes encoding insulin and insulin-like peptides; genes encoding heat shock factors; genes encoding catalases; stress-response genes; and metabolic genes. The invention further relates to methods for identifying and using agents, including small molecule chemical compositions, antibodies, antisense nucleic acids, and ribozymes, that regulate, e.g., enhance, adult lifespan via modulation of aging associated proteins; as well as to the use of expression profiles, markers, and compositions in diagnosis and therapy related to lifespan extension, life expectancy, and aging. The present invention also relates to gene therapy involving lifespan associated genes.

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/20247

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) : A01K 67/033; C07H 21/04; C12N 15/63, 1/21
US CL : 435/252.3, 69.1, 320.1; 800/8; 536/23.2, 235

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.3, 69.1, 320.1; 800/8; 536/23.2, 235

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
Please See Continuation Sheet

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y —	US 5, 945, 330 A (HILLMAN et al) 31 August 1999 (31.08.1999), column 27, line 20 to column 32, line 57; column 37 line 18 to column 40, line 9.	30-33
X, P —	KIM, S. HTTP://C. Elegans: Mining The Functional Genomic Landscape Nature Reviews. September 2001, Vol. 2, pages 681-689, especially 682-687.	30-31, 44-45, 47-52
Y, P —		1-29, 32-43, 46
Y —	US 2002/0007496 A1 (ROTHMAN et al) 17 January 2002 (17.01.2002), page 2, par. 13-17; page 3, par. 32 to page 8, par. 67.	1-35, 44-52

Further documents are listed in the continuation of Box C.

See patent family annex.

Special categories of cited documents:		"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier application or patent published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search
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INTERNATIONAL SEARCH REPORT

International application No.

PCT/US02/20247

Box I Observations where certain claims were found unsearchable (Continuation of Item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claim Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claim Nos.: 1-3, 18, 19, 22, 32, 33 and 39 because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically: the claims contains sequences that are not in compliance with the PCT sequence listing requirements (i.e application lacks CRF, RSL, CD submission).

3. Claim Nos.: 41 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of Item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest The additional search fees were accompanied by the applicant's protest.
 No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

PCT/US02/20247

Continuation of B. FIELDS SEARCHED Item 3:

MEDLINE, CAPLUS, BIOSIS, NATURE PUBLISHING GROUP, PNAS, NUCLEIC ACID RES, USPATENT, PGPUB, DERWENT

search terms: ageing, aging, C. elegans, caenorhabditis elegans, rna-interference, mai, longevity, p53, ma library, age related genes, microarray, in vitro, cynthia kenyon, javier apfeld, andrew dillin, delia garigan, ao hsu, josh lehrer graiwer, coleen murphy, university of california, hsf polypeptide, method of (detecting or identifying or modulating) compound that modulates (ageing aging)